한국농화학회지 제 41 권 제 1 호(1998)
Agricultural Chemistry and Biotechnology
Vol. 41, No. 1, pp. 53~59(1998)

Effects of Osmoprotectants on the Growth and Nitrogenase Activity of *Rhizobium* and *Azospirillum* under Osmotic Stress

Young Ju Choi* and Sang Wan Gal

*Department of Food and Nutrition, Silla University, Pusan 616-736, Department of Microbiological Engineering, Chinju National University, Chinju 660-758, Korea

Abstract : The *Rhizobium* and *Azospirillum* spp. were isolated from the root nodules of several leguminous plants and rhizosphere of various paddy rice varieties. The growth of the nitrogen-fixing strains isolated was largely inhibited in yeast extract-mannitol medium (AMA) containing 0.6 M NaCl. In response to osmotic stress, the nitrogen-fixing strains accumulate intracellular free glutamate. The growth and nitrogenase activity of *Rhizobium* and *Azospirillum* were increased by addition of osmoprotectants such as proline, glycine betaine, and glutamate during salt stress. Glycine betaine was the most effective among exogenous osmoprotectants tested. In the absence of sodium chloride, nitrogenase activity seem to be slightly decreased by the presence of the proline or glycine betaine. These results revealed that nitrogenase activity was repressed by fixed nitrogens such as proline or glycine betaine.
(Received January 14, 1998; accepted February 12, 1998)

Introduction

Cellular adaptation to osmotic stress is a major biological process that protects organisms against the lethal effects of dehydration due to high osmotic stress of environment. Osmoregulation is of great significance in agriculture and soil microorganism growth, since water is the critical limiting factor in crop productivity and microorganism growth.

Salinity is an other form of water-related stress responsible for major crop losses worldwide, therefore it has become an ever-increasing problem in semiarid and irrigated agriculture.[1,2]

Plant and microorganisms have evolved a variety of mechanisms for adapting to osmotic stress. The mechanism which bacteria have evolved to cope with osmotic stress in the environment is the intracellular accumulation of certain small organic molecules such as proline, glycine betaine, glutamate, and others.[3-8] These compounds prevent damage from cellular dehydration by balancing osmotic strength of the cytoplasm with that of the environment.[7] Recent experiments suggest that osmoprotectants may influence protein structure and stability.

Rhizobium and *Azospirillum* are nitrogen fixing soil bacteria with great agricultural importance. Most *Rhizobium* strains which nodulate important crops such as soybean, pea, and clover are very sensitive to salt.[9,10] *Azospirillums* are associated with the roots of various plant.[11,12] The genus *Azospirillum* was initially described with two species, *Azospirillum lipoferum* and *Azospirillum brasilense*.[13] Recently, a new type species, *Azospirillum amazonense* was proposed mainly on the basis of phenotypic data. These bacterial strains can enhance the growth of the plant by the production of plant growth prompting substance or nitrogen fixed by the bacteria can be transferred to the plant.[14]

The metabolic activities of microorganisms vary considerably with changes in their growth environments. Environmental changes are most likely to affect the intracellular concentration of the low molecular weight pool constituents. Under conditions of salt stress, gram-negative bacteria that is highly sensitive to changes in growth conditions accumulates predominantly glutamate in the intracellular amino acid pool,[15] whereas gram-positive bacteria accumulates intracellular proline in response to salt stress. However, several kinds of environmental stresses including salinity decrease inoculum viability, nitrogen fixing rate and crop yield. The studies of the biochemical and physiological bases salt tolerance in the diazotrophs are needed to improve host plant performance in saline environment.

In this study, we screened the natural salt tolerant *Rhizobium* and *Azospirillum* from a variety of leguminous plants and paddy rice, and isolated salt tolerant *Acacia* rhizobia sp 86 which can grow yeast extract-manmitol medium (AMA) with 1.4 M NaCl. We investigated the effects of osmoprotective substances such as proline, glycine betaine, and glu-

Key words : osmoprotectant, nitrogenase activity, intracellular free amino acid
*Corresponding author

tamate on the growth and nitrogenase activity of *Rhizobium* and *Azospirillum* under osmotic stress. This work was also undertaken to determine whether nitrogen fixing bacteria accumulate the intracellular amino acid in response to various NaCl concentrations.

Materials and Methods

Bacterial strains

The bacterial strains used in this study are described in Table 1.

Media and culture conditions

Rhizobium was grown in yeast extract-mannitol medium (AMA) containing 10 g of mannitol, 0.5 g of yeast extract, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.5 g of K_2HPO_4, and 4.88 mg of $FeCl_3 \cdot 6H_2O$ per liter. *Azospirillum* was cultured in Congo red medium (RC) containing 5 g of malic acid, 0.5 g of yeast extract, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 0.015 g of $FeCl_3 \cdot 6H_2O$, 4.8 g of KOH, and 15 m*l* of 1:400 aqueous solution of Congo red per liter, respectively. The pH was adjusted to 6.8 with 2N KOH and the medium was autoclaved at 121°C for 15 min.

The nitrogen-free semisolid malate medium[17](NFB) was used for maintenance and *in vitro* acetylene reduction assay of *Azospirillum*. Minimal media used for the determining of proline and free amino acid was M9 and minimal latate mediun medium,[18] and solid media were obtained by the addition of 15 g of agar per liter. Cycloheximide was routinely added to all media at a concentration of 25 mg/m*l* to avoid fungal contamination.

Plant nutrient solution contained (per liter) 0.88 g of $K_2SO_4 \cdot 2H_2O$, 0.12 g of $MgSO_4 \cdot 7H_2O$, 0.34 g of $CaSO_4 \cdot 2H_2O$, 0.125 mg of $CaCl_2 \cdot 6H_2O$, 0.016 g of Fe-EDTA, 0.17 g of $Na_2HPO_4 \cdot 12H_2O$, 10.0 g of sucrose, and 1.0 m*l* of micronutrient solution (g/*l*) consisting of KCl, 3.73; H_3BO_3, 1.55; $MnSO_4 \cdot H_2O$, 0.85; $CuSO_4 \cdot 5H_2O$, 0.13; $(NH_4)MoO_{24} \cdot 4H_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$; pH 7.0. *Rhizobium* and *Azospirillum* were grown aerobically at 28°C and, respectively 37°C. The osmotic strength of the medium was mediated by the addition of sodium chloride.

Table 1. Bacterial strains used in this study

Strain	Source or reference
Bradyrhizobium japonicum 110	Nitragin
Rhizobium ftridii USDA192	Yelton *et al.*[16]
R. meliloti 102F51	Nitragin
R. leguminosarum 897	Johnston and Beringe[6]
R. trifoli	Nitragin
Azospirillum lipoferum KY6	This study
A. lipoferum SK13	"
Acacia rhizobia sp86	"

Isolation of salt tolerant diazotrophs

Salt tolerant *Rhizobium* and *Azospirillum* were screened from the root nodules of several leguminous plants and roots of various paddy rice varieties by the isolation of colony grown in AMA and RC medium containing different concentrations of sodium chloride.

Determination of growth rates

Bacterial cells were cultured in AMA and RC medium. *Rhizobium* and *Azospirillum* were grown at 28°C and 37°C in a rotary shaker in Erlenmeyer flasks, respectively. Growth was followed by measuring the absorbance at 420 nm. Growth curve data were plotted on semi-logarithmic paper and the mean generation times were obtained from the exponential phases of the growth curve.

Measurement of intracellular free amino acid

Intracellular free amino acid was measured by the method of Csonka.[5] The bacterial cells were grown until late log phase (approximate density of 5×10^8 cells/m*l*). Then, 4ml of the culture was rapidly filtered in Millipore filters (0.45 μm pore size). The filters were placed into 10 m*l* of 70% (v/v) ethanol containing 100 *n*mole of D-norleucine as internal standard, and the cells were extracted at room temperature for 20 min with occasional gentle agitation. The cell debris was removed by centrifugation and the ethanol extract evaporated to dryness at 60°C under a stream of N_2. The residue was taken up in 0.2 M sodium citrate buffer (pH 2.2) and was analyzed by amino acid autoanalyzer (LKB 4150).

Nitrogenase activity assay

Acetylene reduction activity of intact root nodules was measured by the methods of Cho *et al.*[19] and specific activity is defined as *n*mol ethylene produced per hour per plant. The number of nodules were assayed from the plant

Table 2. Assay condition of gas chromatography for acetylene reduction activity determination

Item	Condition
Column	Strainless steel column (0.3 × 2 cm) Packed with Porapark N (100-120 mesh)
Detector	Flame ionization detector
Column temp	70°C
Detector temp	100°C
Carrier gas (N_2)	80 ml/min
Chart speed	10 mm/min

after measuring acetylene reduction activity. Whole-cell nitrogenase activity of the *Azospirillum* was measured by the acetylene reduction procedure.[20] Cells cultured in NFB medium for 24 h at 37°C were sealed with serum stopper and acetylene was injected into the culture to give 12% of acetylene pressure. The cultures were incubated for 2 h at 37°C and the ethylene produced was analyzed by gas chromatography.[21] The assay conditions of gas chromatography for acetylene reduction activity determination are listed in Table 2.

Results and Discussion

Salt tolerance and osmoregulatory amino acid

Rhizobium spp. were isolated from the root nodules of each host plant using AMA medium containing sodium chloride. *Azospirillum* spp. were isolated from the rhizosphere of paddy rice using Congo red medium (RC) containing sodium chloride. Salt tolerant test was done by the isolation of colony growth in AMA and RC medium containing different concentrations of sodium chloride (Table 3 and Table 4). The growth of most nitrogen fixing strains was largely inhibited at 0.6 M NaCl. However, *Acacia* rhizobia sp86 isolated from Acacia root nodules was grown up to 1.4 M NaCl. The fast-growing *Rhizobium* revealed higher salt tolerance than slow-growing *Rhizobium*. In contrast, nitrogen-fixing activity of the later was greater than that of the former. The major intracellular accumulation in *Rhizobium* and *Azospirillum* under osmotic stress of sodium chloride was determined to be glutamate. These results were similar to those of other gram negative bacteria.[15,22] However, it has recently been demonstrated that

Table 3. Salt tolerance of rhizobacteria spp. isolated from various leguminous plants

Strain	Host plant	Salt concentration			Source
		0.4	0.6	1.0M NaCl	
Cowpea rhizobia SY3	*Vigna unguiculata* (cowpea)	+	-	-	This study
Rhizobium. Phaseoli SY12	*Phaseolus vulgaris* (bean)	+	-	-	This study
R. fredii SY6	*Glycine max* (soybean)	+	+	-	This study
Bradurhizobium japonicum SY8	*Glycine max* (soybean)	-	-	-	This study
R. meliloti SY17	*Medicago* spp. (alfalfa)	+	+	-	This study
R. trifoli SY13	*Trifolium* spp. (clover)	+	-	-	This study
R. lupini SY21	*Lupinus* spp. (lupine)	+	-	-	This study
Lotus rhizobia sp11	*Lotus* spp.	+	+	-	This study
Acacia rhizobia sp86	*Acacia* spp.	+	+	+	This study
R. fredii USDA192		+	+	-	Yelton *et al.*[16]
R. meliloti 102F51		+	+	-	Nitragin
R. trifoli		+	-	-	Nitragin
R. leguminosarum 897		+	-	-	Johnston and Beringe[6]
Bradyrhizobium japonicum 110		-	-	-	Nitragin

* Salt tolerant test was done by the isolation of colony grown in AMA medium containing NaCl.

Table 4. Salt tolerance of *Azospirillum* spp. isolated from various rice varieties

Strain	Associated plant	Colony formation				Source
		0.2	0.4	0.6	0.8M NaCl	
Azopirillum lipoferum						
KY6	Kaya (rice)	+	+	-	-	This study
KY7	Kaya (rice)	+	+	-	-	"
Sp71	Sampung (rice)	+	+	+	-	"
SK13	Samkang (rice)	+	+	+	-	"
SK16	Samkang (rice)	+	+	+	-	"
SK27	Samkang (rice)	+	+	-	-	"
Azospirillum brasilense						
SK17	Samkang (rice)	+	+	+	-	"
Azospirillum brasilense						
SP7	Digitaria	+	-	-	-	Lab collect
SP107	Wheat	+	+	-	-	Lab collect
Azospirillum lipoferum						
SP59	Sorgum	+	+	-	-	Lab collect
UQ1618	Maize	+	+	-	-	Lab collect

* *Azospirillum* was isolated from the roots of rice using Congo red medium (RC) containing sodium chloride.

E. coli, S. typhimurium, and related enterobacteria can accumulate proline, betaine or both to high intracellular concentrations in response to high osmotic pressure.[23,24]

Effects of osmoprotectants on growth rates and nitrogenase activity

Exogenous osmoprotectants such as proline and glycine betaine are known to stimulate the growth rates of different enteric bacteria during osmotic stress.[5] The effects of proline and glycine betaine on growth rate of *Rhizobium fredii* USDA192 (Fig. 1) and *Azospirillum lipoferum* KY6 (Fig. 2) in various sodium chloride concentration of less than 0.6 M NaCl, but the addition of proline and glycine betaine at a concentration of 1 mM enhanced markedly the growth rate, and at low or in the absence of salt stress, growth of these strains is not significantly affected by the presence of osmoprotectants.

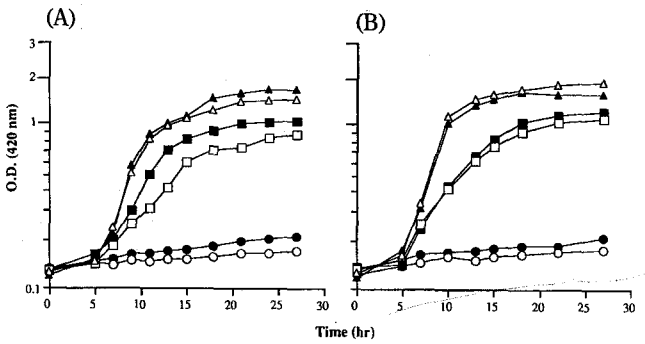

Fig. 1. Effects of proline [A] and glycine betaine [B] on the growth of *A. lipoferum* KY6 in different NaCl concentration. Optical density is plotted as a function incubation time. Osmoprotectant concentration : 1 mM.
△—△; 0.0 M NaCl, ▲—▲; 0.0 M NaCl + 1 mM Proline [A] or Betaine [B], □—□; 0.3 M NaCl, ■—■; 0.3 M NaCl + 1 mM Proline [A] or Betaine [B], ○—○; 0.6 M NaCl, ●—●; 0.6 M NaCl + 1 mM Proline [A] or Betaine [B]

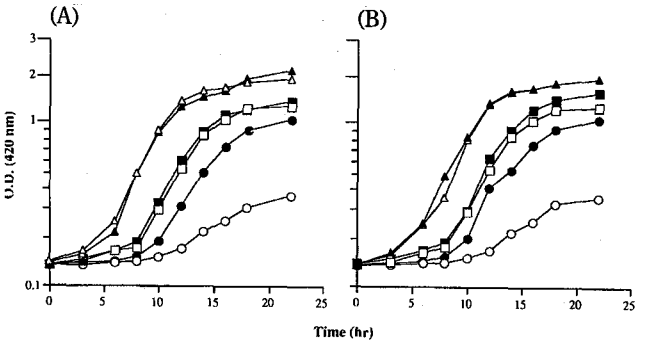

Fig. 2. Effects of proline [A] and glycine betaine [B] on the growth of *R. fredii* USDA 192 in different NaCl concentration. Optical density is plotted as a function incubation time. Osmoprotectant concentration : 1 mM.
△—△; 0.0 M NaCl, ▲—▲; 0.0 M NaCl + 1 mM Proline [A] or Betaine [B], □—□; 0.3 M NaCl, ■—■; 0.3 M NaCl + 1 mM Proline [A] or Betaine [B], ○—○; 0.6 M NaCl, ●—●; 0.6 M NaCl + 1 mM Proline [A] or Betaine [B]

Osmoprotectants such as proline and glycine betaine were increased the biological activities of *Rhizobium* and *Azospirillum* when they were added to growth medium. These results mean that through proline and glycine betaine like glutamate were not be synthesized or accumulated, but can play a key role as osmoprotectants in many diverse species, including higher plant. Most bacteria do not synthesize betaine *de novo* and are dependent on uptake of betaine or its precursor, choline.[25] By the addition of these osmoprotectants, microorganisms not only restores turgor pressure across the cell membrane but can also protect enzymes from inactivation at high ionic strength.[7,26]

Effects of exogenous osmoprotectants (proline, glycine betaine, and glutamate) on nitrogen fixations of *Rhizobium* and *Azospirillum* are showed in Table 5 and Fig. 3. Nitrogenase activities of *Rhizobium* under conditions of less than 200 mM of salt stress were found to be markedly stimulated by addition of 1 mM osmoprotectants. However, in the presence of 0.2 M NaCl, the nitrogenase activity was not detectable. These results reveal that leguminous plants can not grow up to at a concentration that *Rhizobium* can grow. There are remarkable similarities between bacteria

Table 5. Effects of osmoprotectants on *Rhizobium* under various conditions of salt stress

Strain (host)	Salt conc.(mM) osomoprotectant[1]	Acetylene reduction activity (nmol C_2H_4/hr/plant)[2]	Number of nodule per plant[2]
Bradyrhizobium	0	416 ± 115	12.5 ± 3.5
japonicum 110	0 + Glutamate	313 ± 97	14.2 ± 4.7
(soybean)	0 + Proline	364 ± 89	15.4 ± 6.1
	0 + Betaine	426 ± 109	16.7 ± 5.9
	100	238 ± 93	10.3 ± 4.5
	100 + Glutamate	299 ± 86	12.5 ± 5.4
	100 + Proline	380 ± 113	13.5 ± 6.7
	100 + Betaine	407 ± 107	14.2 ± 5.9
	200	-	-
	200 + Glutamate	-	-
	200 + Proline	-	-
	200 + Betaine	-	-
Rhizobium	0	41.3 ± 12.4	8.3 ± 3.2
meliloti 102F51	0 + Glutamate	37.5 ± 11.2	7.2 ± 4.3
(alfalfa)	0 + Proline	35.6 ± 9.5	8.6 ± 4.5
	0 + Betaine	40.3 ± 10.5	9.4 ± 4.7
	100	30.5 ± 11.5	6.9 ± 3.5
	100 + Glutamate	39.6 ± 10.7	7.8 ± 5.7
	100 + Proline	40.5 ± 8.4	8.4 ± 4.7
	100 + Betaine	40.4 ± 12.6	8.8 ± 3.7
	200	-	-
	200 + Glutamate	-	-
	200 + Proline	-	-
	200 + Betaine	21.3 ± 17.3	5.1 ± 3.7

[1]Osmoprotectant concentration : 1mM.
[2]Means ± SE

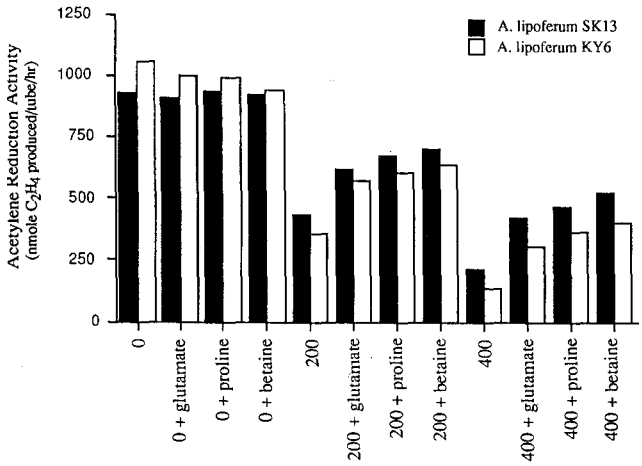

Fig. 3. Effects of osmoprotectants on nitrogenase activity of *Azospirillum lipoferum* KY6 and SK13 in free living states under salt stress. *In vitro* acetylene reduction activity was measured as C_2H_4 produced per tube after 24 h culture in semisolid nitrogenase free malate (NFB) medium and 2 h incubation under 12% (V/V) actylene at 37°C. Osmoprotectant concentration : 1 mM. Salt concentration : 0, 200, 400 mM sodium chloride.

and plants in their cellular response to osmotic stress. Among osmoprotectants tested, glycine betaine was most effective in symbiotic association with soybean.

In *Azopirillum*, nitrogenase activity under salt stress was also markedly stimulated by addition of 1 mM osmoprotectants. In the presence of 0.4 M NaCl, the nitrogenase activity of *A. lipoferum* KY6 in the absence of exogenous glycine betaine was 138 *n*mol C_2H_4/hr/tube, compared to a value of 401 *n*mol/hr/tube in the presence of 1 mM glycine betaine, but in the absence of sodium chloride, nitrogenase activity seems to be slightly decreased by the presence of the proline or glutamate, presumably because glutamate or proline serve as a source of supply of fixed nitrogen. In general, nitrogen fixing organisms were repressed the nitrogenase activity by fixed nitrogen.[27,28] In contrast to *E. coli*, the osmoprotectants can function in *Rhizobium* as a carbon and nitrogen source in the absence of salt stress, but under salt stress, these osmoprotective compounds can only play an important role as an osmoprotectant.[29]

Intracellular amino acid levels under salt stress.

In order to verify whether enhanced osmotolerance is correlated with increased intracellular amino acid levels, we determined the free amino acid contents of salt tolerant *Acacia* rhizobia sp86 and *A. lipoferum* spp. grown in the presence of a various NaCl concentrations (Table 6 and Table 7). In salt tolerant *Acacia rhizobia* sp86, the intracellular level of free glutamate was increased to 5-fold greater in the presence of 1.4 M NaCl than in the control, whereas proline was

Table 6. Intracellular free amino acid changes in *Acacia* rhizobia sp86 under sodium chloride stress

Amino acid	Amino acid conc. (μmol/g of cell, dry weight)		
	0.0	0.8	1.4
Asp	0.45	1.96	1.03
Thr	0.54	2.05	1.86
Glu	2.21	5.94	11.86
Pro	6.49	5.15	7.04
Gly	6.26	5.93	1.22
Ala	0.49	0.81	2.07
Val	0.55	1.23	2.25
Met	0.28	0.13	0.30
Iso	0.13	0.20	1.54
Leu	0.23	0.21	1.67
Tyr	0.19	NDb	0.22
Phe	ND	0.07	0.99
Trp	1.35	2.01	1.58
Lys	0.46	2.13	1.44
	Glutamate to total amino aicd (%)		
	11.30	21.10	33.90

Table 7. Intracellular free amino acid changes in *Azopirillum* under sodium chloride stress

Amino acid	Amino acid conc.(μmol/g of cell, dry weight)					
	A. lipoferum KY6			*A. lipoferum* SK13		
	0.0	0.2	0.4	0.0	0.3	0.6M NaCl
Asp	0.31	0.48	0.36	0.16	0.37	0.47
Thr	0.44	0.54	0.45	0.54	0.43	0.68
Ser	0.41	0.68	0.63	0.53	0.51	0.99
Glu	0.61	0.75	0.85	0.69	1.57	1.89
Pro	0.32	0.41	0.40	0.46	0.75	0.79
Gly	0.17	0.04	0.23	0.06	0.13	0.04
Ala	0.27	0.16	0.08	0.10	0.19	0.15
Val	0.61	0.67	0.55	0.75	0.66	0.84
Met	0.32	0.32	0.30	0.39	0.36	0.36
Iso	0.36	0.42	0.40	0.42	0.40	0.52
Leu	0.63	0.84	0.79	0.96	0.88	1.08
Tyr	0.38	0.30	0.26	0.40	0.35	0.31
Phe	0.59	0.57	0.52	0.72	0.67	0.75
Trp	0.58	0.42	0.39	0.58	0.49	0.49
Lys	1.00	0.93	0.78	1.22	1.18	1.03
	Glutamate to total amino acid (%)					
	8.7	10.1	12.3	8.7	12.6	13.6

detected in small amounts but its concentration did not appear to be affected by the presence of 1.4 M NaCl. These results were consistent with the finding of Hua *et al.*[30] who showed increased glutamate levels under salt stress in the *Rhizobium* sp.WR 1001. *Salmonella typhimurium* also accumulates glutamate in response to osmotic stress.[3] Tempest *et al*[22] first reported accumulation of glutamate in cells grown with osmotic stress. In utilization of carbon sources, salt tolerant *Acacia* rhizobia sp86 used a wider range than the other *Rhizobium* (data not shown).

Similar result was also observed in *Azospirillum lipof-*

erum KY6 and SK13. The intracellular free glutamate level of *A. lipoferum* KY6 was lower than that of *A. lipoferum* SK13, and the salt tolerance of the later was greater than that of the former. These results suggest that salt tolerance is correlated with increased intracellular free glutamate level. This phenomenon may be described as a part of osmoregulatory response of the organisms.

References

1. Epstein, W., G. A, Cunninghan, D. B. Kelley, R. W. Kingsbury, J. D. Norlyn, D. W. Rush, and A. F. Wrona (1978) Crop production in arid and semiarid regions using saline water, P. 15-23. A report for the National Science Foundation Applied Science and Research Application. National Science Foundation, Washington, D. C.
2. Rains, D. W. (1979) Salt tolerance of plants : Strategies of biological systems, P. 47-67. In A. Hollander, J. C. Aller, E. Epstein, A. San Pietro, and O. R. Zaborsky (ed.), The biosaline concept. Plenum Publishing Crop., NewYork.
3. Botsford J. L., M. Alvarez, R. Nichols (1994) Accumulation of glutamate by *Salmonella typhimurium* in response to osmotic stress. *Appl. Environ. Microbiol.* **60**, 2568-2574.
4. Cairney, J., I. R. Booth, and C. F. Higgins (1985) *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. *J. Bacteriol.* **164**, 1218-1223.
5. Csonka, L. N. (1981) Proline over-production results in enhanced osmotolerance in *Salmonella typhimurium. Mol. Gen. Genet.* **182**, 82-86.
6. Johnston, A. W. B., and J. E. Beringer (1976) Pea root nodules containing more than one *Rhizobium species. J. Bacteriol.* **161**, 882-887.
7. Le Rudulier, D., A. R. Strom, A. M. Dandeaker, L. T. Smith, and R. C. Valentine (1984) Molecular biology of osmoregulation. *Science* **224**, 1046-1068.
8. Noel, K. D., and W. J. Brill (1980) Diversity and dynamics of indigenous *Rhizobium japonicum* population. *Appl. Environ. Microbiol.* **40**, 931-938.
9. Steinborn, J., and R. J. Roughley (1975) Toxity of sodium and chloride ions to *Rhizobium* spp. in broth and peat culture. *J. Appl. Bacteriol.* **39**, 133-138.
10. Upchurch, R. G., and G. H. Elkan (1977) Comparison of colony morphology, salt tolerance, and effectiveness in *Rhizobium japonicum. Can. J. Microbiol.* **23**, 1118-1122.
11. Dobereiner, J., and J. M. Day (1976) Associative symbioses in tropical grasses : Characterization of microorganisms and dinitrogen fixing sites. P. 518-538. In W. E. Newton and C. J. Nyman (ed.), Proceeding of the 1st International Symposium on N_2 Fixation, Washington State University Press, Pullman.
12. Haahtela, K., T. Wartiovaara, V. Sundman, and J. Skujins (1981) Root associated N_2 fixation (acetylene reduction) by *Enterobacteriaceae* and *Azospirillum* strains in cold-climate spodosols. *Appl. Environ. Microbiol.* **41**, 203-206.
13. Tarrand, J. J., N. R. Krieg, and J. Dobereiner (1978) A Taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genes, *Azospirillum* gen. nov., and *Azospirillum brasilense* sp. nov. *Can. J. Microbiol.* **24**, 967-980.
14. Okon, Y., S. L. Albrecht, and R. H. Burris (1976) Factors affecting growth and nitrogen fixation of *Spillum lipoferum. J. Bacteriol.* **127**, 1248-1254.
15. Measures, J. C. (1975) Role of amino acids in osmoregulation of nonhalophilic bacteria. *Nature* (London) **257**, 398-400.
16. Yelton, M. M., S. S. Yang, S. A. Edie, and S. T. Lim (1983) Characterization of an effective salt tolerant, fast-growing strain of *R. japonicum. J. Gen. Microbiol.* **139**, 1537-1547.
17. Baldani, V. L. D., and J. Dobereiner (1980) Host plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem.* **12**, 433-439.
18. Ratti, S., B. Curti, G. Zanetti, and E. Galli (1985) Purification and characterization of glutamate synthase from *Azospirillum brasilense. J. Bacteriol.* **163**, 724-729.
19. Cho, M. J., M. S. Yang, H. D. Yun, Z. R. Choe, Y. L. Choe, and K. Y. Kang (1985) Genetic engineering of biological nitrogen fixation and its application to agronomy. *Kor. J. Appl. Microbiol. Bioeng.* **13**, 75-82.
20. Mayfield, C. I., and R. L. Aldworth (1974) Acetylene reduction by non-symbiotic bacteria in artificial soil aggregates amended with glucose. *Can. J. Microbiol.* **20**, 877-881.
21. Burris, R. H. (1972) Nitrogen fixation assay method and techniques. *Methods Enzymol.* **24**(B), 415-431.
22. Tempest, D. W., and J. L. Meers (1970) Influence of environment content and composition of microbial free amino acid pools. *J. Gen. Miocrobiol.* **64**, 171-185.
23. Le Rudulier, D., and L. Bouillard (1983) Glycine betaine, an osmotic effect in *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae. Appl. Environ. Microbiol.* **46**, 152-159.
24. Perroud, B., and D. Le Rudulier (1985) Glycine betaine transport in *Escherichia. coli* : Osmotic modulation. *J. Bacteriol.* **161**, 393-401.
25. Molenaar, D., A. Hagting, H. Alkema, A. J. M. Driessen (1993) Characteristics and osmoregulatory roles of uptake systems for proline and glycine betaine in *Lactococcus lactis. J. Bacteriol.* **175**, 5438-5444.
26. Le Rudulier, D., and R. C. Valentine (1982) Genetic engineering in agriculture : Osmoregulation. *Trends Biochem. Sci.* **7**, 431-433.
27. Enzo, G., and M. Bazzicalupo. (1985) Effect of nitrogen compound on nitrogenase activity in *Azospirillum brasilense. FEMS Microbiol. Lett.* **28**, 35-38.
28. Okon, Y. (1985) *Azospirillum* as a potential inoculant for agriculture. *Trends Biotechnol.* **3**, 223-228.
29. Le Rudulier, D., and T. Bernard (1986) Salt tolerance in *Rhizobium* : A possible role for betaine. *FEMS Microbiol. Rev.* **39**, 67-72.
30. Hua, S. T., V. Y. Tsai, G. M. Lichens, and A. T. Noma (1982) Accumulation of amino acids in *Rhizobium* sp. strain in response to sodium chloride salinity. *Appl. Environ. Microbiol.* **44**, 135-140.

질소고정균의 성장과 질소고정력에 대한 osmoprotectant의 영향

최영주* · 갈상완(신라대학교 식품영양학과, 진주산업대학교 미생물공학과)

초 록 : 수종의 두과작물과 수도에서 분리한 *Rhizobium* 및 *Azospirillum*들의 내염성을 조사하고 salt stress에 의하여 유도되는 균주의 생리적 특성 및 식물이나 미생물에서 osmoprotectant 로 작용하는 proline, glycine betaine 및 glutamate가 질소고정균의 생육이나 질소고정력에 미치는 영향을 연구하였다. 분리된 대부분의 질소고정균들은 0.6 M NaCl 농도에서 생육이 현저히 감소되었지만 *Acacia* rhizobia sp86 은 1.4 M NaCl 농도에서도 생육이 가능하였다. Osmotic stress에 의하여 증가되는 intracellular 유리아미노산은 *Rhizobium* 및 *Azospirillum*에서 glutamate 였으며, 특히 *Acacia* rhizobia sp86은 salt stess에 의하여 5배정도 glutamate를 축적하였다. Osmoprotectant (proline, glycine betaine, glutamate)를 배지내에 1 mM 첨가함으로서 salt stress에 의하여 감소되는 질소고정균의 생육과 질소고정력을 방지하였으며, glycine betaine이 가장 효과적이었다.

찾는말 : osmoprotectant, nitrogenase activity, intracellular free amino acid

*연락저자