CHAPTER IV

DISCUSSION

Saline soil was shown as one of the important ecological factor that affected the loss of symbiotic nitrogen fixation efficiency (Nutman, 1958; Andrew,1977) Soil of high salinity not only induced a disturbance in nodulation process but also inhibited growth of most strains of Rhizobium. (Sprent, 1984). Howerver, tolerance to saline condition of Rhizobium varied from species to species including within species as well. (Rai, et. al., 1982) In the present study, we attempted to isolate rhizobia of ability to grow under high salt concentration and successively done by using sibling selection. Four strains of an effective nodulation named as P_1, P_5, P_{19} and P_{21} were isolated from R. phaseoli, possessed an ability to grow in the medium supplemented with NaCl up to 0.3 M. Viewed from all of the physiological properties studied such as ability to nodulate Phaseolus vulgaris, or to grow in YM. and YM. plus 0.3 M NaCl, or orthers, all strains isolated possessed a minor difference in phenotypic properties but a closed similarity in genotypic ones. For example, some significant variations shown in the number of nodules per plant did not cause a significant difference in ARA, plant wet weight and plant height (Table 2). Furthermore, a minor difference in value revealed in duration of log phase, generation time, growth yield occurred in growth for both under salted and no salted supplementation (Table 3). Of course, there was a remarkable difference in properties compared between salted and no salted condition in each strain, however difference in group of values within the same column

Table 8a. Comparison of P_{19} properties to the WT's

Strain and cultivation condition	Surface Antigen	Nodulation efficiency	% GC Content
WT in YM	fast growing type	control	61.2
P_{19} in YM	same as WT	no significant difference compared with the WT	61.2
P_{19} in YM + 0.3 M NaCl	same as WT	ND.	ND.

ND. = not done

Table 8b. Comparison of P_{19} properties to the WT's

Strain and cultivation condition	Morphology		Duration taken in log phase (d.)	Generation time (hr.)	Maximal growth (KU.)	Rate of O_2 consumption (µmole/min/mg prot.)	ATPase activity (µmole/min/mg prot.)	Initial rate of Na^+ efflux (nmole/min/mg prot.)
	SEM.	TEM.						
WT in YM	control	envelope:control nucleus : control	0.5	2	350	6.4	2.2	0.2
P_{19} in YM	same as control	envelope:distortion nucleus : same as control	1.2	4.5	300	4.8	2.7	1.0

obtained under salted and no salted condition weresimilar (Table 3). We, therefore, believed that properties of P_{19} should be appropriate for a discussion in the role of salt tolerance, since P_{19} was the strain used for most of the studies in this thesis. Properties of P_{19} were therefore summarized for the convenience of discussion purposes. (Table 8a, b, c).

Eventhough P_{19} showed a slow growing type of Rhizobium in growth character (Table 3) but its efficiency to nodulate the host plant was similar to the WT (Table 2). Strain of ability to nodulate the same plant with an equal efficiency should be a derivative of each other. And the result of fluorescent surface antigen antibody obtained from both WT and P_{19} did support the result of nodulation. In addition, the identical values in % GC content of the chromosomal DNA of WT and P_{19} did help confirmation that the slow growing type of growth character should be in situ nature hindered within the genotype of P_{19}. In fact, the % GC content for most rhizobia ranged from 59-65%; with a distinction between the fast growers of 59.1-63.1%, and a slow frowers of 61.5-65.5% (Vincent, 1977).

Because all of the strains isolated were spontaneous mutants of the same WT, therefore, a defection occurred in the chromosomal DNA should be the consequence of a point mutation. (Miller, 1972)

However, if all the properties of WT and mutant P_{19} grown under the same condition were the point of comparison, (Table 8b) we could conclude that mutation caused by a single base change revealed as the salt tolerant genotype provided a pleiotropic effect found in all properties studied. For example, the initial rate of the Na^+ efflux of the mutant P_{19} was 5 folds increased when compared to that of the WT . There was a minor

Table 8c. Comparison of properties of P_{19} cells grown under with and without salt.

Strain and cultivation condition	Morphology		Duration taken in log phase (d.)	Generation time (hr.)	Maximal growth (KU.)	Rate of O_2 consumption (μmole/min/mg prot.)	ATPase activity (μmole/min/mg prot.)	Initial rate of Na^+ efflux (ηmole/min/mg prot.)
	SEM.	TEM.						
P_{19} in YM	same as WT	envelope: distortion nucleus : same as WT	1.2	4.5	300	4.8	2.7	1.0
P_{19} in YM + 0.3 M NaCl	same as WT	envelope: distortion nucleus : more compact	5	19.0	210	10.0	2.7	1.7
Quotient ratio of P_{19} in YM + 0.3 M. NaCl / P_{19} in YM			4.2	4.2	0.7	2.1	1.0	1.7

difference revealed among values related to the energy yielding process. The value of O_2 consumption, and of the Ca^{+2}, Mg^{+2} ATPase activity in the mutant were slighty lower and higher than those found in the WT. Because the quotient values obtained from the WT to the mutant in terms of KU and rate of O_2 consumption were similar, ie.,equal to 1.2 and 1.3 respectively, (Table 8b). It could therefore speculate that a lowering of O_2 consumption caused a minor change in growth yield. However, why the generation time of the mutant was approximately double longer than that of the WT was unexplianable. One could only speculate that a small distortion seen in the mutant envelope might cause the delay in its generation time.
The phenomenon that there was a growth delay in the salt tolerant mutant was also reported by previous reporters. For example, salt tolerant strains which isolated from R. trifolii and R. meliloti by Steinborne and Roughly (1975) and strains which isolated from R. leguminosurum by Rai (1983) showed a reduction in growth rate similar to that established by ours. However, no one persued the involvement in the bio-chemical role of the isolated strains as we did for ours. Above all, we could conclude that the increase in the rate of Na^+ efflux was one of the role which allowing our mutants to multiply in a medium of high salt concentration.

Table 8c, summarized the properties of mutant P_{19} studied from cells grown with and without salt. Except the activity of Ca^{+2}, Mg^{+2} ATPase, all properties registered showed a remarkable influence in the consequence of an addition of high salt during cell growth. Viewed from the quotient ratio of various properties derived from P_{19} grown in YM+0.3 M NaCl per P_{19} grown in YM values, as shown in Table 8c, would help seeing the unexplainable phenomena as the consequence of high salt. For example, in the presence of high salt concentration, the rate of O_2 consumption was double increased whereas the yield of cell growth was one-third declined.

In addition, 1.7 folds increase in the value of Na^+ efflux should suggest the direct role of Na^+ efflux to confront the saline stress in the medium, but why there occurred at the same time,a very long-delay in the cell growth.

Figure 9, 10, 11 illustrated the TEM micrographs of mutant cells, cultivated in the presence of salt, the denser character of nucleoplasm portion including the irregularity seen in membrane envelope were obviously due to the effect of salt. Vreeland, et. al., (1983) reported the same phenomenon in Halomonas elongata, a halophilic bacterium strain which could cultivate in a rather wide range of salt concentration (0.05 - 3.4 M NaCl). They postulated that there was a leakage of intracellular water due to the external environment in the process of osmolarity adjustment caused by the higher level of the external concentration of salt than that of the intracellular one. Thus, the remaining amount of intracellular water would be less than that obtained under unsalted condition. Another words, an optimal activity of water was essential to keep a regular viability to the cell. Therefore, a decrease in water activity would disturb to the whole metabolic process. For example, the dense character of nucleoplasm might be the result of a stronger binding between DNA and DNA or DNA and RNA and the retroeffect of this result was directly seen in the extended generation time. If this explanation was accepted, it would probably encase the phenomenon that, why the increase level of O_2 consumption of mutant cells resulted in the decrease in the maximal growth yield, as established in the Table 8c.

In an attempt to find a relation between the energy yielding process and the rate of Na^+ efflux, the inhibitory effects of FCCP and DCCD to the initial rate of Na^+ efflux were persued and the results were shown in Table 7.

Owing to DCCD, an inhibitor of Ca^{+2} Mg^{+2} ATPase did not significantly deviate the rate of Na^{+} efflux whereas FCCP, an inhibitor of the proton gradient, did remarkably ; we, therefore, concluded that respiratory energy, not the coupling energy should be the direct source that drive the flux of Na^{+} out from the cell. Such of this conclusion was known long before in case of E. coli (West and Mitchell, 1974). We were well awared that the unequality in the minus effect of FCCP occurred as shown in table 7 caused this data less accurate in terms of quantitative evaluation. Way to improve the result is to design the assay of Na^{+} in terms of second instead of minute as we did in case of ours. However, our results were similar to those of the uptake experiments as reviewed by Kaback and Hong in "Membranes and Transport" CRC, Critical Reviews in Microorganisms (1973) vol. 2. 333 -374.

Conclusion

Spontaneous mutants of salt tolerant properties, isolated by an application of a sibling selection were studied to a certain extent. Properties of these isolated mutants could be summarized as follows:

1. Growth properties were changed from a fast growing to a slow growing type of Rhizobium.

2. Cell envelope around inner membrane portion as observed from TEM was changed from a smooth to a rough surface. Cultivation in salted supplemented medium caused more irregularity seen in the inner membrane including a denser and more compact structure of nucleoplasm.

3. Energy yielding process in terms of O_2 consumption rate was fluctuated according to the cultivating medium whereas the coupling state of energy in terms of ATPase activity was steady regardless of the cultivating medium.

4. A markedly increase in rate of Na^+/H^+ efflux was observed especially in cells cultivated under salted condition. FCCP not DCCD completely demolished the efflux of Na^+.

5. Inspite of there altered properties, all the salt tolerant strains still maintained the ability to nodulate the host plant with a comparable efficiency.

6. % GC content of the chromosomal DNA, cell surface antigen, SEM study supported the evidence that the phenotypic properties changed were resided in the same genotype being revealed as salt tolerant mutants of ability to multiply in 0.3 M NaCl supplemented medium.