CHAPTER - VIII

CONCLUSIONS
Iron is the fourth most abundant element in the Earth's crust, however under aerobic conditions and at neutral pH it is present as a component of insoluble minerals (1). Since, this element is an essential micronutrient involved in many biochemical processes most of the aerobic and facultative anaerobic organisms have evolved a mechanism of high affinity iron transport for its acquisition whether this is from soil, the phyllosphere, marine or fresh water environments, or from animal host tissues. This mechanism involves synthesis by microorganisms of low molecular weight iron chelating compounds, termed as siderophores which are excreted in the immediate environment so as to solubilize and assimilate this critically important metal ion (2,3).

Rhizobia live in the soil or engage in a symbiosis with a suitable legume. Under symbiotic conditions also iron requirement by rhizobia is crucial since iron is required at a number of stages in the biological fixation of nitrogen (4). Thus, irrespective of the habitat rhizobia needs to have an efficient iron transporting machinery. The first report on siderophore production in rhizobia was published in 1984 by Smith and Neilands (5). Thereafter, quite a few reports have shown siderophore production by different rhizobia. But till date exact mechanism of high affinity uptake system in rhizobia is not clear. With this background, present investigation was mainly concentrated on high affinity iron transport by cowpea Rhizobium GN1 under ex planta conditions.

The cowpea Rhizobium GN1 was found to produce siderophores under Fe-limited condition as was evidenced by growth on chrome-azurol S (CAS) agar and reaction of supernatant of Fe-limited culture with CAS-assay solution. Arnow's test showed the siderophore to be of catecholate type. Thin layer chromatography and UV spectrophotometric scanning helped in detection of 2,3-dihydroxybenzoic acid (DHBA) and 3,4-DHBA in the siderophore extract. The 2,3-DHBA appeared to be conjugated with alanine and lysine. The reports on nature of the siderophores from amongst different species of rhizobia show a wide variation in the
structures (4). But presence of 2,3-DHBA in siderophore has been reported in Rhizobium leguminosarum (6) and R. trifolii (7). The siderophore of cowpea Rhizobium GN1 was an efficient iron chelator and could promote the growth of cowpea Rhizobium GN1 in a medium containing synthetic iron chelators like bipyridyl or ethylenediamine tetraacetic acid (EDTA). Stoichiometric studies indicated that siderophore equivalent to 2 moles of DHBA was required for chelation of one mole of Fe.

At 10 μM concentration of Fe in the growth medium siderophore could not be detected in the medium suggesting that this compound is essentially produced only under Fe-limited conditions. The synthesis of the siderophore by cowpea Rhizobium GN1 under Fe-deficient condition was growth associated and maximum levels of siderophore in the growth medium were obtained around 20-22 h of growth.

Though iron was the prime regulator of siderophore biosynthesis, the levels of the siderophore produced were affected by nutritional and environmental factors. Maltose and succinate were found to be best suited as 'C' sources for the maximum siderophore production whereas urea and sodium glutamate were best 'N' sources which yielded maximum levels of the siderophore. With citrate as a 'C' source even traces of siderophore could not be detected. Further bioassay with ferric citrate and uptake of Fe-citrate showed that citrate can act as an iron transporting compound in cowpea Rhizobium GN1. Ferric-citrate can serve as a source of iron in number of organisms including E. coli, Pseudomonas aeruginosa and Bradyrhizobium japonicum (8-10). As expected, phosphate levels in the growth medium affected the siderophore production and maximum siderophore production was obtained with phosphate concentration of 0.6 g/L. Some of the metal ions also had an important role to play in siderophore biosynthesis. Magnesium was essential for siderophore production as well as growth whereas zinc and copper could cause increase in the levels of siderophore. Metal ions like cobalt and chromium inhibited siderophore production drastically.
In vitro enzymatic biosynthesis of 2,3-DHBA from chorismic acid using cell-free extract of *Rhizobium* GN1 suggested that this organism follows the same pathway for synthesis of 2,3-DHBA as was reported in *Aerobacter aerogenes* 62-I (11). It was also observed that presence of aromatic amino acids in the growth medium, which can affect the levels of chorismate in the organism, caused decrease in siderophore production.

Increased aeration rate and alkaline pH of the medium resulted in increased siderophore production. The contention of M. Guerinot that one of the reasons for failure in detection of siderophore in *Bradyrhizobium japonicum* could be that the strains were probably not tested under the appropriate nutritional conditions (4) appears to hold true as results in cowpea *Rhizobium* GN1 also showed that nutritional as well as environmental factors strikingly affect the siderophore production.

The siderophore of cowpea *Rhizobium* GN1 was actively involved in the iron uptake in this organism. Studies with uptake of $^{55}$Fe-siderophore complex showed that the cells priorly grown under Fe-limited condition were very efficient in iron uptake as compared to the cells priorly grown under Fe-sufficient conditions. The outer membrane protein (OMP) profiles of Fe-limited (-Fe) cells and Fe-sufficient (+Fe) cells of the organism revealed that OMPs of -Fe cells have two proteins / polypeptides with approximate molecular weights 80 kDa and 76 kDa which were absent or lowly expressed in +Fe cells. As the receptors for Fe-siderophore complexes are expressed under Fe-limited conditions (12), these two proteins from cowpea *Rhizobium* GN1 may have a role to play in siderophore mediated iron uptake. It was also observed that OMPs from -Fe cells could bind $^{55}$Fe-siderophore complex more efficiently than the OMPs from +Fe cells. Autoradiographic studies also showed that $^{55}$Fe-siderophore complex could bind to both the iron repressible polypeptides (i.e. 80 kDa and 76 kDa). These results explain why -Fe cells were more efficient in siderophore mediated iron
uptake than +Fe cells. Thus, along with siderophore production, the presence of cognate receptor protein in the outer membrane is essential for the functioning of high affinity iron transport in cowpea Rhizobium GN1. Saturation kinetics of siderophore mediated iron uptake also suggested that this uptake was receptor mediated.

The siderophore mediated iron transport in cowpea Rhizobium GN1 was energy requiring process. Inhibition of iron uptake by compounds like potassium cyanide, sodium azide, 2,4-dinitrophenol and arsenate indicated that the energised membrane is required for the transport. Here the required energy could be attained through electron transport or by hydrolysis of ATP.

Standard / authentic 2,3-DHBA and 3,4-DHBA could promote the iron uptake but they were less efficient than the siderophore. Thus, it is quite likely that conjugation of amino acids to DHBA results in an efficient iron chelator.

Once the cell receives Fe-siderophore complex it should have some mechanism by which iron is released from the complex. It was observed that cowpea Rhizobium GN1 possess constitutive iron reductase activity. This enzyme reduces ferric iron to ferrous iron which is subsequently released from siderophore. The enzyme iron reductase which required flavins alongwith NADH as reductant for its activity was mainly localised in the periplasmic space. In vitro studies revealed that the enzyme was sensitive to oxygen and probably required uncomplexed sulfhydryl groups in the enzyme for the activity. It was also found that iron reduction was not associated with cell respiration. The enzyme iron reductase could act on substrate like Fe-siderophore, ferric citrate, ferric-dihydroxybenzoic acid, ferric chloride and ferrioxamine. But the maximum enzyme activity was obtained with Fe-siderophore. Thus in cowpea Rhizobium GN1, the enzyme iron reductase appears to be involved in removal of iron from Fe-siderophore complex.
Mutants of cowpea Rhizobium GN1 were obtained by chemical mutagenesis and transposon mutagenesis. The mutant GN1-M obtained by nitrosoguanidine treatment was siderophore non-producing whereas the mutant P4-5 obtained by transposon (Tn5) mutagenesis was siderophore over producing. Inability of GN1-M to produce siderophore made it vulnerable to iron deficient conditions. It could not grow in a low iron medium or a medium containing synthetic iron chelator. Thus, this observation showed the necessity of the siderophore to the organism to overcome iron limitation. Further analysis of GN1-M showed that it could use siderophore from the wild type indicating that the receptor proteins in this mutant were not affected. The OMP profile of this mutant also showed the presence of two iron regulated / repressible polypeptides (80 kDa and 76 kDa) as were observed in wild type. This supported our earlier speculation that these two polypeptides may play a role of receptor (or component of receptor) for Fe-siderophore complex. The analysis of the other mutant i.e. P4-5 showed that though this mutant produced more siderophore it was less efficient in iron uptake than the wild type. This could be the reason for its decreased growth under iron limited conditions. But this mutant was not completely inhibited by iron limitation as was observed with GN1-M. Amongst N$_2$-fixing organisms mutants with phenotype similar to P4-5 have been reported in Azospirillum brasilense (13) whereas siderophore non-producing mutants have been successfully isolated in Rhizobium meliloti (14, 15) and Azotobacter vinelandii (16, 17).

To survive under natural conditions the organism has to successfully compete with other microflora in the surrounding. Better nutrient acquisition, resistance to the environmental factors and elimination of competitors from the surroundings by production of antibiotics or bacteriocins are some of the mechanisms known for the survival of a given species in the soil environment. Antagonism mediated through siderophores has been reported in number of organisms like Pseudomonas sp. (18) and Azospirillum spp. (19, 20). The siderophore of cowpea
Rhizobium GN1 when tested for antimicrobial activity, if any, showed it to be inhibiting the growth of a few organisms like *Salmonella* sp., *Serratia* sp., *Azotobacter* sp., *Staphylococcus aureus* and some Gram positive soil isolates. Among fungi only *Chaetomium* sp. and *Rhizopus* sp. were found to be sensitive to the siderophore. The mechanism of growth inhibition of the test cultures by siderophore was through chelation of iron. Hence, it appears that under natural soil conditions where iron may act as a growth limiting factor, the siderophore of cowpea *Rhizobium* GN1 will have a critical role to play in the survival of this organism.

Microbial siderophores which increase and regulate iron availability in the rhizosphere are suggested to be involved in the iron nutrition of plants (21), but the possibility whether the host legume itself may benefit from rhizobial siderophore production has not been explored (4). An iron inefficient variety of peanut was studied for evaluating possibility of involvement of siderophore of cowpea *Rhizobium* GN1 in iron nutrition. It was found that the peanut plants, when grown hydroponically with the siderophore of cowpea *Rhizobium* GN1, showed increased growth and chlorophyll content as compared to plants grown with Fe alone. The siderophore was effective in plant growth promotion when used at concentrations lesser than the concentration of Fe used, indicating that it might function as a shuttle agent, solubilizing and supplying Fe to the plant. Similar results were obtained with desferrioxamine B, a hydroxamate siderophore. The pattern of results with the siderophore of cowpea *Rhizobium* GN1 and desferrioxamine B was almost same. This indicated that the mechanism by which both these compounds promote iron nutrition in peanut was same. Roots of peanut showed iron reducing activity which was more pronounced when plants were grown under Fe-limited conditions suggesting that it may have some role to play in iron acquisition.

The present investigation thus bring us to conclude that cowpea *Rhizobium* GN1 has very well developed high affinity iron uptake system
which involves catecholate siderophore. The siderophore of this organism not only helps in overcoming the problems of iron limitation but also has a role to play in rhizosphere. The ability to produce siderophore will act as an added asset to survive under soil conditions as well as it will contribute to the plant growth promoting activities of rhizobia.
REFERENCES


