CHAPTER II

MATERIALS AND METHODS
Organisms Used:

Cowpea Rhizobium GN1 was isolated locally from the root nodules of peanut (*Arachis hypogea*). The culture was maintained on yeast extract mannitol agar (YMA) containing congo red.

The bacterial and fungal strains used for antimicrobial activity assays were partly from departmental culture collection and partly isolated from soil. The bacterial cultures were maintained on nutrient agar whereas fungal cultures were maintained on Sabouraud's agar.

Culture Conditions:

**Media:**

Yeast extract mannitol medium as described by Vincent (1) was used. The composition in g/L distilled water: Mannitol 10, Yeast extract 1, NaCl 0.1, MgSO$_4$·7H$_2$O 0.2, K$_2$HPO$_4$ 0.5, 1% congo red solution 0.25 ml. The medium was solidified using 2.5% agar (pH 6.8 - 7.0 self adjusted).

Ashby's mannitol broth (g/L): Mannitol 10, Sodium glutamate 2, NaCl 0.1, MgSO$_4$·7H$_2$O 0.2 and K$_2$HPO$_4$ 0.5. When needed, the medium was solidified with 2.5% agar (pH 6.8 - 7.0 self adjusted).

Nutrient agar (g/L): Peptone 10, Meat extract 3, NaCl 5 and agar 25 (pH 7.0).

Sabouraud's agar (g/L): Glucose 40, Peptone 10, NaCl 5 and agar 25 (pH 5.5)

**Condition of growth:**

Cowpea Rhizobium GN1 was grown in Ashby's mannitol broth (AMB) in 250 ml Erlenmeyer flask on rotary shaker (180 rpm) at 28 ± 2°C. A 2% inoculum was used and the culture was harvested during late log phase.
The carbon and nitrogen sources in AMB were altered as and when mentioned in the text during optimisation studies. For the preparation of iron deficient medium all the chemicals used were of analytical grade and the medium was prepared in either deionised or double glass distilled water. All the glasswares were cleaned by using chromic acid followed by 6N HCl and then rinsed thoroughly with double distilled water. For the removal of impurities of iron, the medium was extracted with 8-hydroxyquinoline (0.25% in chloroform) followed by extraction with plain chloroform so as to remove traces of Fe-quinates and unreacted hydroxyquinoline (2). When effect of metal ions on siderophore production was studied, the medium was prepared as follows.

The carbon and nitrogen sources were dissolved in double glass distilled water; pH was adjusted to 7.5 and medium was autoclaved at 15 lbs pressure for 15 mins. after addition of 0.5% alumina. The medium was cooled and filtered to remove alumina. Other components of the medium were added, pH was adjusted to 7.0 and the medium reautoclaved at 10 lbs pressure for 20 mins.

Detection and Estimation of Siderophore:

Chrome azurol-S (CAS) agar plates: The method described by Schwyn and Neilands (3) was followed. To prepare 1 litre of the blue agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM FeCl₃ in 10 mM HCl). While stirring, this solution was slowly added to 72.9 mg hexadecyl trimethyl-ammonium bromide (HDTMA) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved at 15 lbs pressure for 15 mins.

In a separate flask 100 ml 10X MM9 salts, 30.24 g Pipes and 12.0 g of 50% (w/w) NaOH solution were added and dissolved in 750 ml double distilled water so as to get pH 6.8, Then 25 g agar was added and it was autoclaved at 15 lbs pressure for 15 mins. After cooling to 50°C, 30 ml Casamino acids (10%), 10 ml glucose (20%), 5 ml L-glutamic acid
(10% neutralised) and 2.5 ml biotin (0.02%) were added as sterile solutions. The dye solution was added last with agitation to achieve mixing without generation of foam. Each plate received about 30 ml of blue agar. For detection of siderophore, culture was spread on the plates and colonies developed were observed for any yellowish orange halo around the colony.

**CAS assay solution**: The composition of CAS solution used for the detection of siderophore in the supernatant of culture grown in Fe-limited medium was essentially same as described by Schwyn and Neilands (3). A 6.0 ml volume of 10 mM HDTMA solution was placed in a 100 ml flask and diluted with water. A mixture of 1.5 ml iron (III) solution (1 mM FeCl₃ in 10 mM HCl) and 7.5 ml 2 mM aqueous chrome azurol-S solution was slowly added under stirring. A 4.307 g quantity of anhydrous piperazine was dissolved in water and 6.25 ml of 12 M HCl was carefully added. The above two solutions were mixed together in volumetric flask and made upto 100 ml to get CAS assay solution, which was stored in polyethylene bottle kept in dark. The detection of siderophore was done by mixing equal volumes of CAS assay solution and the culture supernatant. Colour change from blue to yellowish orange was indicative of presence of siderophore.

For estimation of catecholate siderophore, Arnow's method (4) was used. In 1 ml of culture supernatant, 1 ml of 0.5 N HCl was added followed by addition of 1 ml of nitrite-molybdate reagent (10 g% each of sodium nitrite and sodium molybdate in distilled water); after 5 mins of waiting 1 ml of 1N NaOH was added and pink colour developed was read at 500 nm. Authentic 2,3-dihydroxybenzoic acid was used as standard.

Estimation, in the culture supernatant, of hydroxamate compounds, if any was done by Gibson and Magrath method (5).
Isolation and Characterization of Siderophore:

After desired incubation in Fe-limited medium, the cells were harvested by centrifugation (10,000 X g for 15 min) in Sorvall RC5C centrifuge. The supernatant was acidified to pH 2.0 and extracted with equal volume of ethyl acetate thrice. The ethyl acetate layers were collected, pooled together and evaporated to dryness. The residue (siderophore extract), thus obtained was analysed by thin layer chromatography on silica gel G using solvent system benzene: toluene: acetic acid (2:2:1 v/v/v). The plates were developed by spraying Hathway's reagent (6) (Ferric chloride 0.3 g; potassium ferricyanide 0.3 g in 100 ml distilled water). The Rf values of blue spots were measured. Authentic sodium salicylate, 2,3-dihydroxybenzoic acid (DHBA), 3,4-DHBA, 3,5-DHBA and catechol were used as standards. The compounds present in the siderophore extract were separated and collected by preparative TLC.

For the detection of conjugated amino acids, the siderophore extract was acid hydrolysed with 6N HCl at 100°C for 1 h followed by reextraction with ethyl acetate and then neutralization of aqueous phase with 1N NaOH. The aqueous phase was subjected to paper chromatography for detection of amino acids (solvent system butanol: acetic acid: water, 4:1:5 v/v/v). The chromatogram was developed with ninhydrin reagent (7).

The reextracted siderophore sample was subjected to preparative TLC and the bands corresponding to standard 2,3-DHBA and 3,4-DHBA were scraped out. The scraped out silica were suspended in neutral methanol and then filtered. The compound thus, dissolved in methanol was subjected to UV spectrophotometric scanning on Shimadzu spectrophotometer UV-260. Authentic phenolates similarly dissolved in neutral methanol were used as standards.
Bioassay of siderophore: Ashby’s mannitol agar containing synthetic iron chelators like bipyridyl (800 μM) or ethylenediamine tetraacetic acid (2 mg/ml) was prepared. In petri plates containing this medium, the culture (10⁷ cells/ml) was surface spread. Wells of 8 mm diameter were made in plates with cork borer. The siderophore extract dissolved in sterile saline (0.8% NaCl, pH 8.0) was then added in the well. Control wells contained sterile saline (pH 8.0). Plates were incubated at 4°C for 3 h for better diffusion, followed by 24 h incubation at 28 ± 2°C and then examined for zone of growth exhibition around the wells containing siderophore.

Bioassay for ferric-citrate was carried out in the same way.

Antimicrobial activity assays: A known volume (20 ml) of sterilized Luria-agar was poured in petri plates. A suspension of about 10⁷ cells/ml (10⁷-10⁸ spores/ml in case of molds) was then surface spread. Wells of 8 mm diameter were made with a cork borer. The method of siderophore addition in the wells and incubation of plates was similar to one described above for bioassay. Here the plates were observed for inhibition of growth around the well containing siderophore extract.

Stoichiometric analysis of Fe-siderophore complex: The ability of siderophore to remove iron from Fe-CAS-HDTMA complex was used to determine the moles of iron removed from the Fe-CAS-HDTMA complex by using known concentration of the siderophore. Details are described in Chapter IIIA.

Iron Uptake Assays:

⁵⁵FeCl₃ was obtained from Bhabha Atomic Research Centre, Bombay, India. ⁵⁵Fe-siderophore complex was formed by mixing ⁵⁵FeCl₃ with approximate 4 mole excess of siderophore. The concentration of ⁵⁵Fe (either free Fe or Fe-siderophore complex) in the uptake buffer was 1
μM. For uptake studies bacterial cells were grown in either iron-deficient or iron-sufficient Ashby's mannitol broth. Cells from mid-log phase were collected by centrifugation (at 10,000 X g for 15 min.), washed twice with the uptake buffer (5 mM morpholinoethane sulphonate buffer, pH 7.0) and suspended in the same buffer containing either only $^{55}$Fe or $^{55}$Fe-siderophore complex. The uptake study was carried out in 250 ml flask containing 50 ml uptake buffer with approximately $10^9$ cells per ml. At intervals of 5 min, 1 ml of the samples were removed and filtered through membrane filters (0.45 μm pore size) which had been previously soaked in the uptake buffer for 30 min and the bacteria immediately washed with 5 ml of 0.5% sodium thioglycollate. Filters were dried and counted in Ria Luma (Lumac/3M bv, The Netherlands) in liquid scintillation counter (LKB Rackbeta Spectral Scintillation Counter). All values were corrected for binding of the iron complexes to the filter.

Saturation kinetics of iron uptake was studied by carrying out uptake studies using different concentrations of Fe-siderophore complex (0.025 μM to 5.0 μM) in the uptake buffer.

To study / determine the energy dependence of iron uptake, inhibitors like potassium cyanide (2 mM) or sodium azide (10 mM) or 2,4-dinitrophenol (0.4 mM) or arsenate (4 mM) were added in the uptake buffer (containing cells) 5 mins before initiating uptake with addition of $^{55}$Fe-siderophore complex.

**Isolation of Crude Envelope Proteins:**

Cowpea Rhizobium GN1 was grown in the AMB medium at 27 ± 2°C under shaking conditions and the cells were harvested from the exponential phase culture by centrifugation (10,000 X g at 10°C for 15 min). For iron limited culture the above medium was prepared in double glass distilled water and extracted with 8-hydroxyquinoline prior to inoculation. For iron sufficient culture the above medium was
supplemented with 100 μM Fe. Rest of the procedure for preparation of crude cell envelope was same as described below. Unless otherwise stated, all operations were done at 0-4°C. The harvested cells were washed twice in 0.9% sodium chloride and suspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.0). The cells were disrupted at 0°C by ultrasonication for 3 min (6 X 30S) using Vibronics Ultrasonic Processor P2. The disrupted cells were centrifuged for 10 min at 1000 X g to remove unbroken cells. In the supernatant, KCl was added to final concentration of 0.2 M. This supernatant contained unseparated membranes which were pelleted out by centrifugation for 30 min at 1,30,000 X g at 4-6°C. The pellet was washed with 50 mM Tris-HCl buffer (pH 8.0) and recentrifuged for 30 min. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 6.8) and used as crude envelope preparation for further studies.

Isolation of Outer Membrane Proteins :

a) Density gradient centrifugation : The outer and inner membranes from the crude envelope fraction were separated by sucrose density gradient centrifugation (70-30% w/v). The crude envelope fraction was loaded on the sucrose gradient and centrifuged at 1,80,000 X g for 4 h. After centrifugation the tubes were punctured from the bottom and fractions collected. The fractions were analysed for 2-ketodeoxyoctonate content (marker for outer membrane) and NADH oxidase activity (marker for cytoplasmic membrane).

b) Extraction with Triton X-100 : Outer membrane proteins were isolated from the washed crude envelopes by treatment with Triton X-100 (2% and 2.5% in Tris-HCl buffer, 0.1 M, pH 7.8 with 10 mM magnesium chloride) as described by Schnaitman (8). The Triton X-100 extracted membrane referred here as 'Outer Membrane', were collected by centrifugation at 1,00,000 X g for 60 min, washed in 0.1 M Tris-HCl buffer (pH 7.8) containing 10 mM magnesium chloride and resuspended in about 2 ml of 0.06 M Tris-HCl buffer (pH 6.8).
Isolation of Periplasmic and Cytoplasmic Proteins:

Periplasmic proteins were isolated by two different methods viz. EDTA-lysozyme treatment described by Glenn and Dilworth (9) and freeze-thaw method described by Paoletti et al. (10).

In case of EDTA-lysozyme treatment the cells, harvested after growth by centrifugation (10,000 X g for 15 min), were suspended in 5 ml of 50 mM Tris (pH 8.0) - 20% (w/v) sucrose - 2 mM EDTA - 2 mg of lysozyme per ml and incubated at room temperature for 30 min. The cells were pelleted by centrifugation at 4°C. The supernatant was considered as 'periplasmic fraction' and checked for alkaline phosphatase (marker for periplasmic proteins) and malate dehydrogenase (marker for cytoplasmic proteins). The pellet was further suspended in 50 mM Tris-HCl (pH 8.0), followed by brief sonication. The unbroken cells were removed by centrifugation and the supernatant was subjected to ultracentrifugation (1,80,000 X g for 30 min) to remove cell envelopes. The supernatant, thus collected was considered as 'cytoplasmic fraction'.

In freeze-thaw method the washed cell pellet obtained from exponentially growing culture was suspended in 50 mM K-phosphate buffer (pH 6.8) and kept at -20°C overnight. The sample was then thawed and cells were pelleted out by centrifugation at 10,000 X g for 15 mins. The supernatant fluid was collected and considered as 'periplasmic fraction'.

Enzyme Assays:

Enzymatic synthesis of 2,3-DHBA: It was studied by using cell-free extract of cowpea Rhizobium GN1 grown under Fe-limited conditions in AM broth. The assay was carried out as described by Young et al. (11). The reaction mixture for enzymatic synthetic of 2,3-DHBA was a final volume of 1 ml containing 50 μmoles Tris-HCl buffer (pH 8.0), 2 μmoles chorismate, 2 μmoles NAD, 5 μmoles magnesium chloride and 0.2 ml of crude cell free extract (approx. 25 mg/ml protein). After incubation for
30 min, the reaction mixture was acidified with 0.5 ml of acetate buffer (0.75 M, pH 4.0), extracted with ethyl acetate and 2,3-DHBA estimated spectrophotometrically.

The enzyme activity was expressed as μmoles of 2,3-DHBA formed.

Chorismate required for above assay was isolated from **Aerobacter aerogenes** 62-1 as described by Gibson (12).

_Ferri-siderophore reductase / iron reductase_: Reduction of iron by cell-free extract was measured by using Ferrozine (ε<sub>562</sub> = 28,000 M<sup>-1</sup> cm<sup>-1</sup>) as described by Dailey and Lascelles (13). The reaction system described by Dailey and Lascelles was modified and the enzyme assay was carried out under anaerobic conditions (nitrogen atmosphere). The reaction mixture (final volume 2.2 ml) contained 1.3 ml Tris-HCl buffer (25 mM with 25% glycerol, pH 8.0), 0.1 ml NADH (15 mM), 20 μl FAD (1 mM), 0.2 ml ferrozine (10 mM), 0.4 ml Fe-siderophore (1 mM), 20 μl magnesium chloride (100 mM) and 0.2 ml enzyme preparation i.e. crude cell-free extract (0.5 - 2 mg protein). All the reagents used were flushed with nitrogen (oxygen free) before use. Before addition of enzyme in the cuvette, the cuvette with rest of the reagents was also thoroughly flushed with nitrogen for 3-4 mins. Blank was prepared in the same way except without ferrozine. After addition of enzyme preparation, increase in O.D. at 562 nm was recorded. The activity of the enzyme was recorded as nmol Fe(II) formed per minute per mg protein. To check effect of reductants on enzyme activity, NADH was replaced by succinate (50 mM, 0.1 ml) or NADPH (15 mM, 0.1 ml). Similarly for studying effect of flavins, FAD in the reaction system was omitted or replaced by FMN. When different iron complexes were examined as substrates for enzyme activity, Fe-siderophore in the above system was replaced by that particular iron complex. The iron complexes were prepared by reacting FeCl<sub>3</sub> with compounds like 2,3-DHBA, the siderophore of cowpea **Rhizobium** GN1 and desferrioxamine.
B (obtained from Sigma Chemical Company). To study effect of metal ions, magnesium was replaced by other metal ions (at 1 mM concentration).

**Malate dehydrogenase (EC 1.1.1.37)**: This was assayed as described by Ochoa (14). The assay system contained 100 umoles K-phosphate buffer (pH 7.2), 0.2 umoles NADH and 0.4 umoles of oxaloacetate. Appropriate quantity of enzyme was added. One unit of enzyme was defined as the amount of enzyme which brings about a change in 0.01 O.D. at 340 nm.

**Alkaline phosphatase (EC 3.1.3.1)**: It was assayed by measuring the rate of increase of the A$_{420}$ in a 3 ml test solution containing 0.1 M Tris-HCl (pH 8.6), 0.4 M MgCl$_2$, 1.2 mg of p-nitrophenyl phosphate and 100 ul of enzyme solution (15). A unit of enzyme activity was defined as the amount of enzyme which brings about a change in 0.01 O.D. at 340 nm.

**NADH oxidase (EC 1.6.99.3)**: Method described by Osborn et al. (16) was followed. 0.1 ml aliquot of enzyme (membrane fraction) was added to 2.9 ml of mixture containing 0.05 M Tris-HCl (pH 7.5), 0.12 mM NADH and 0.2 mM dithiothreitol. The rate of decrease in absorbance at 340 nm was measured spectrophotometrically. Enzyme activity was expressed as umoles (of NADH oxidased) per min per ml.

**Chemical and Analytical Methods**:

**2-ketodeoxyoctonate (KDO)**: The estimation was done according to the method described by Karkhanis et al. (17). The assay was as follows: 1 ml of 0.2N H$_2$SO$_4$ was added in a sample containing KDO. It was heated at 100°C for 30 min. cooled and centrifuged at 6000 X g for 5 min. 0.5 ml of the clear supernatant was taken in another tube and 0.25 ml of 0.04 M HIO$_4$ (periodic acid) in 0.125N H$_2$SO$_4$ was added, vortexed and allowed to stand at room temperature for 20 min. In this 0.25 ml of 2.6%
NaAsO₂ in 0.5N HCl was added, vortexed and again allowed to stand until the brown colour disappeared. Then 0.5 ml of 0.6% thiobarbituric acid was added, vortexed and heated at 100°C for 15min. While hot, 1 ml of dimethyl sulphoxide was added, the mixture was cooled to room temperature and absorbance was read at 548 nm against the blank treated as above without KDO. The molar extinction coefficient calculated thus, is 44625 mol⁻¹·cm⁻¹.

Protein estimation: Protein estimation was done by Lowry's method (18) but in case of outer membrane proteins, method described by Tolbert et al. (19) was followed.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): Electrophoresis of membrane proteins was done in SDS-polyacrylamide gel (10%) as described by Lugtenberg et al. (20). For comparison between outer membrane proteins obtained by sucrose density gradient centrifugation and Triton X-100 treatment, a gradient gel (5-20%) electrophoresis was done as described by Walker (21). Protein bands were stained with Coomassie Brilliant Blue R-250 and destained with 10% v/v methanol - 10% v/v acetic acid solution.

Autoradiography: The outer membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane by electroblotting as described by Towbin et al. (22). The nitrocellulose was incubated for 4-6 hours in solution of 3% skimmed milk powder in 10 mM Tris-HCl (pH 7.4) and 0.9% NaCl. The nitrocellulose was then incubated with ⁵⁵Fe-siderophore complex for 3-4 hours. It was then washed thoroughly with distilled water (deionised), the nitrocellulose was then used to develop an autoradiogram using X-ray film.
Fe-siderophore Binding to Outer Membrane Proteins:

The outer membrane proteins were incubated with $^{55}$Fe-siderophore complex at room temperature for 2-3 hours. Then the reaction mixture was filtered through nitrocellulose membrane filter (0.2 μM pore size). The filters were washed extensively with 10 mM Tris-HCl, pH 7.5 and 0.9% saline. The $^{55}$Fe retained on the filter was counted in LKB Rackbeta Spectral Scintillation counter and the results were expressed as percent radioactivity retained on the filter.

Isolation of Mutants Defective in Iron Transport:

Mutagenesis was carried out by using N-methyl N'-nitro-N-nitrosoguanidine as described by Arceneaux et al. (23) and by transposon (Tn5) mediated mutagenesis using E. coli strain harboring pSUP2021 plasmid as described by Marugg et al. (24). The mutagenised culture was plated on Ashby's mannitol agar (AMA) containing 100 μM Fe. The well isolated colonies were grided on Ashby's mannitol agar containing 200 μM bipyridyl as well as CAS agar plates. The colonies which failed to grow on AMA containing bipyridyl or which did not show any yellowish orange halo on CAS plate or showed larger halo around the colony than the wild type were considered as mutants having defect in iron transport and selected for further studies.

Plant Culture:

Seeds of *Arachis hypogea* (obtained from Department of Crop Improvement, Gujarat Agriculture University, India) were surface sterilized with 0.2% HgCl₂ and subsequently washed several times with sterile distilled water. Seeds were germinated in sterile petri plates containing moistened filter paper for about 6-7 days. Seedlings with shoots approximately 4 cm in height were washed with distilled water and transferred to aerated dark-brown glass pots (3 plants per pot) containing 500 ml nutrient solution with or without iron. The nutrient
solution used was modified iron free Hoagland's solution (25) prepared in deionised water or double glass distilled water with following composition: (g/L) Ca(CO$_3$)$_2$ 1.18, KNO$_3$ 0.51, K$_2$HPO$_4$ 0.14, MgSO$_4$ 0.49, H$_3$BO$_3$ 0.0029, MnCl$_2$ 0.0018, ZnSO$_4$ 0.00022, CuSO$_4$ 0.00008, H$_2$MoO$_4$ 0.00002. Addition of iron was done according to the need of experiment. The pH of the medium was adjusted to 7.5 with 0.1 M KOH and 1.0 g/L CaCO$_3$ was added as buffering agent. The pH was monitored daily and corrected to give 7.5 ± 0.1. The medium from pots was replaced at 7 days interval.

**Growth of Plants:**

After germination, the seedlings were transferred to a continuously aerated Fe-free nutrient solution in 500 ml pots. All the treatments of iron, with or without iron chelators, were given subsequently. During iron optimisation studies different concentrations of only Fe were used.

For the study of iron chelators two successive experiments were conducted, each experiment contained 6-8 replications of all the treatments. The treatment of selection was based on results obtained with Fe-optimisation experiments and consisted of combinations of 10 µM Fe with different concentrations (10, 20, 30 and 50 µM) of iron chelators. All experiments involved appropriate control treatment which included corresponding concentrations of Fe but not iron chelators. The plants were grown in growth chamber (with 6 h day and 8 h night cycle). The growth of the plant was monitored for about 40 days and was expressed as increase in shoot length since there was no significant differences in branching and average leaf area of the plants. The height of the plants was measured at intervals according to the type of experiment and needs.
Extraction and Estimation of Chlorophyll Content of Leaves:

The leaves were washed several times with deionised water, midrib was removed from the leaves, and 0.5 g was weighed from each plant. The leaves were placed in plastic bags and kept in cold for few hours till they became turgid. Chlorophyll extraction was done in dark at 10 ± 2°C and estimation as described by Moore (26).

Measurement of Ferric-reducing Activity of Roots:

Plants were placed with their roots in nutrient solution at pH 5.3 with 0.6 mM 2,2'-bipyridyl and 0.4 mM Fe(III)-EDTA at 25°C. The absorption by Fe(II)-bipyridyl complex formed in samples was measured after every 15 min upto 2 hours described by Bienfait et al. (27) [Molar Extinction Coefficient at 520 nm for Fe(II)-bipyridyl is 8650].
REFERENCES


