DISCUSSION
Although iron is the fourth most abundant element in the Earth's crust, it is present as a component of insoluble minerals under aerobic conditions and at neutral pH. Bacterial growth, in turn, depends on the availability of iron, an essential nutrient that participates in many biological processes, including electron transport chains and is a cofactor of enzymes of intermediary metabolism and nitrogen fixation. Therefore, the possession of specialised iron transport may be crucial for bacteria to override the iron limitation imposed by the environment.

One of the most commonly found strategies evolved by microorganisms is the production of siderophores, low molecular weight iron chelators that have very high constants of association for their complexes with iron, and their cognate membrane receptors.

The process of acquisition of iron by rhizobia, collectively referring to *Rhizobium* and *Bradyrhizobium*, both as symbiont and as free-living microorganisms is needed to be understood. During symbiosis rhizobia must acquire iron from their plant hosts and as free-living microorganisms they must compete for iron with other microorganism. Very limited information is currently available regarding iron acquisition in rhizobia; it is also not quite clear if iron plays any regulatory role in the symbiosis, although Bergersen pointed out the importance of iron in root nodules in 1963.

Siderophore production by rhizobia nodulating *Cicer*
... arietinum, a most commonly grown pulse legume in India and nodulated by both *Rhizobium* and *Bradyrhizobium*, was surveyed in the present investigation. Thirteen strains of *Rhizobium* and 9 strains of *Bradyrhizobium* previously isolated from *Cicer arietinum* were screened on CAS-agar medium for the purpose. Absence of growth of most of the *Bradyrhizobium* strains and the *Rhizobium* strain TAL 621 and only scanty growth of *R. meliloti* BICC 604 (Table 5) indicate the toxicity of CAS reagent affecting growth of the strains. Among the strains showing normal growth on CAS-agar medium, 5 produced siderophores as revealed by orange halo zones (Fig. 6) indicating not all the strains could produce siderophore in CAS agar plate. The halo zones surrounding the colonies of the producer strains varied in area (Table 6) indicating differences in the ability of the organisms to produce siderophores.

Since quite a few strains examined were unable to grow and produce siderophore in the CAS-agar plates, a different approach was taken to screen their siderophore producing ability. For that the strains were grown in liquid Complete medium in which all the strains exhibited normal growth. Filtrates from these cultures were assayed with CAS reagent to assess production of siderophore. In this assay also, the strains which exhibited both growth and siderophore production in CAS-agar plates responded positively to CAS-reagent (Table 7). None of the strains furnishing their inability to grow in CAS-agar plates produced siderophore. The data indicate that not all the strains of rhizobia produce siderophores under iron-limiting condition. The
result confirms the observation of Guerinot et al. (1990) whose study revealed that only one out of 20 strains of *B. japonicum* produced a siderophore. The reason for the absence of detectable siderophore in the non-producing strains of *Rhizobium* and *Bradyrhizobium* is unclear, for these bacteria possess an iron-dependent respiratory type of metabolism with an operative tricarboxylic acid cycle (Chakrabarti et al., 1987; Mandal & Chakrabartty, 1993). The inability to detect siderophore production by some of the strains could be due to very low production or the siderophore could be tightly bound to the surface of the bacteria and hence not detectable in supernatants. Another probable reason might be that the strains have taken a long time for production of siderophore and thus the siderophore was not amenable to detection during the period studied. In an earlier study, *Bradyrhizobium* 61A152 was found not to produce any siderophore by Guerinot et al. (1990). However, Carson et al. (1992a) found that the strain excreted and utilised citrate as a siderophore and it took up to 11 days to achieve concentrations of $195 \mu$M citrate, the siderophore of 61A152, in a minimal medium. Carson et al. (1992a) concluded from the observation that the lack of siderophore production reported by Guerinot et al. (1990) by *Bradyrhizobium* 61A76 might only be a problem of detection (Carson et al. 1992a). Guerinot (1991) while discussing the possible reasons of failure in detecting siderophore production by some of the bradyrhizobial strains examined by them hypothesised that the strains might be producing siderophores under natural nutritional conditions only. She maintained that if
the strains were grown in a medium which mimic the nutritional regime of rhizosphere (Buyer et al., 1989), a more valid assessment of a strain's production capabilities in the soil environment would be made. However, from the fact that most of the bradyrhizobial strains examined in this investigation lack the capacity of detectable siderophore production (Table 7), it is possible that having evolved in the acid soils of the tropics where iron is more generally available than in neutral or alkaline soils (Guerinot et al., 1990) the strains lost the capacity to produce siderophore over a long period of evolutionary time without being under selection pressure.

In order to critically assess if the CAS-positive strains produced only one kind of siderophore and if the siderophores produced by the strains were similar, the culture filtrates were analysed by paper chromatography. It was observed that the strain BICC 637 produced siderophores which separated into three bands having $R_F$ values of 0.40, 0.19 and 0.90 respectively. *R. leguminosarum* bv. *viciae* BICC 635 produced two separate bands of siderophores of $R_F$ values 0.23 and 0.13 respectively. The strains BICC 632 and BICC 651 each produced siderophore which yielded only one band having $R_F$ values of 0.82 and 0.80 respectively. *Bradyrhizobium* sp. (*Cajanus cajan*) BICC 616 produced siderophores which separated into two bands of $R_F$ values of 0.3 and 0.14 respectively. So it is evident from the results that some of the strains produced more than one kind of siderophores. As a matter of fact, BICC 637 produced as many as three different kinds of siderophores (Table 8).
There are now a number of reports on the occurrence of siderophores in root nodule bacteria and the siderophores represent a wide range of structures including the unique siderophore, rhizobactin (Smith & Neilands, 1984) along with anthranilic acid (Rioux et al, 1986 a), citric acid (Guerinot et al, 1990) catecholates (Guerinot, 1991) and hydroxamate (Carson et al, 1992 a). Our findings using different assay reagents (Table 9) and also by chromatographic separation of siderophores from the culture filtrates of different strains of root-nodule bacteria (Table 8) add to the growing evidence that rhizobia, like other bacterial species, exhibit strain to strain variation in the ability to produce siderophores (Reigh & O'Connell, 1988). It may be noted that all the 22 strains tested in the present investigation by the method of Rioux et al (1986 a) responded positively (Table 9). This indicated that all the strains produced phenolate type of compounds. But apart from BICC 651, BICC 637, BICC 632, BICC 635 and BICC 616 the other strains did not yield CAS positive reactions and only the strains BICC 651, BICC 637 and BICC 632 yielded positive Arnow reaction showing yellow coloured reaction mixture which turned red in presence of NaOH. CAS is a universal indicator of siderophores irrespective of their chemical structure and Arnow reagent reacts with compounds containing two vicinal phenolic OH groups. A general explanation of the observation then would be that the fractions of the phenolate compounds which did not react positively with Arnow reagent to yield red colour were devoid of vic. diol groups and also were not siderophore in respect of
their function. For characterisation of rhizobial iron acquisition system further studies were conducted with the strain BICC 651, since the strain produced only a single kind of siderophore at a high concentration.

Total iron contents of the media were measured using chromogen ferrozine reagent following the method of Stookey (1970), using deionised water as the control and ferrous ammonium sulphate as the standard. While preparing the standard curve the lower limit of sensitivity of the method was assessed to be 0.3 \( \mu \text{M} \). Iron content of Complete medium deferrated with hydroxyquinoline was undetectable with this method, indicating the iron content of the medium to be below 0.3 \( \mu \text{M} \). Kinetics of growth and siderophore production by Rhizobium strain BICC 651, in the deferrated Complete medium showed that as much as 110 n mol ml\(^{-1}\) siderophore, based on DHBA content, was produced during the late-exponential phase in this medium (Fig.7). Thereupon, however, a sharp decline in the level of siderophore was evident. This could be either due to utilisation of the siderophore by the cells at a fast rate for iron acquisition or that the siderophore became structurally modified or degraded and failed to react with the identifying reagents used. It is very interesting to note that in the later study (Fig. 39 a–e) using over-producing mutants derived from wild type strain BICC 651 the level of siderophore production was found not to decline but it maintained a more or less constant value. So it can be assumed that the factor which act upon siderophore in the wild type strain BICC 651 to bring about a change in the chemical structure of the siderophore,
making it insensitive to Arnow reagent, is perhaps absent in the overproducing mutant strains.

Addition of increasing concentrations of iron to the growth medium and the measurement of siderophore during late-exponential phase indicated that a small amount of iron (0.1 \( \mu M \)) was apparently required in addition to any iron present in the deferrated Complete medium to stimulate optimum production of siderophore but the production was repressed by iron at 50 \( \mu M \) added to the medium. Cells growing in medium with 0.1 \( \mu M \) added iron produced approximately 20-fold more siderophore than those growing with 50 \( \mu M \) added ferric iron (Fig. 8). The higher accumulation of 2,3-DHBA in iron-deficient, relative to iron-sufficient cultures, could result from a requirement of iron for the reaction catalyzed by amino benzoic acid decarboxylase. Repression of rhizobial siderophore synthesis in the presence of iron in the growth medium was also observed by Skorupska et al (1988). It is now the general consensus that low iron induces the siderophore-mediated iron-transport system, whereas high iron represses it. The expression of siderophores and siderophore receptors is controlled by a global repressor protein called Fur (Ferric uptake regulation) (Ernst et al, 1978). Under conditions of iron sufficiency, Fur binds to a highly conserved region called the Fur box upstream of iron-regulated genes and blocks transcription of the iron-regulated genes (Bagg & Neilands, 1987 a; de Lorenzo et al, 1987; Griggs & Konisky, 1989). Under low-iron conditions, repression by Fur is relieved and the genes are transcribed.
Siderophore biosynthesis has been shown to be inhibited by elevated temperatures in several bacteria and fungi. Garibaldi (1971) working on an unidentified fluorescent pseudomonad showed that the organism yielded maximum biomass when grown in a succinate salt medium with iron concentration of 0.1 µg ml\(^{-1}\) at 20°C but 3.0 µg ml\(^{-1}\) of iron was needed for growth at 28°C. This bacterium did not grow in the basal medium at 31°C ever in the presence of 0.01 to 10 µg ml\(^{-1}\) of added iron. The author concluded that the inability of the organism to grow at the higher temperature was due to the loss, by this organism, of its ability to biosynthesise hydroxamate iron transport compounds at temperature of 28°C and above, since supplementation with such compounds promoted growth at 31°C. In the present investigation *Rhizobium* sp BICC 651 exhibited only marginally higher growth and siderophore production at 28°C than that at 37°C (Fig. 9). At 39°C and above growth of the strain was inhibited completely and their growth inhibition could not be alleviated even with 50 µM FeCl\(_3\) supplementation. *Cicer arietinum* is a winter crop, but *Rhizobium* nodulating *Cicer arietinum* being indigenous to the tropical countries like India should have an adaptive measure to survive and nodulate even in higher temperature. Hence, it can be surmised that the bacteria evolved an unique strategy of adaptation of surviving and acquiring much needed iron even in temperature as high as 37°C, so that its siderophore mediated iron acquisition system remains more or less unaffected.

Although, growth of the strain BICC 651 at 39°C was inhibited, however, iron-uptake of the strain was not affected.
markedly at this temperature. In fact uptake of $^{59}\text{Fe}^{3+}$ was found to be operative beyond 40°C (Fig. 22). As such, the inhibition of growth at 39°C is inferred not due to deficiency of uptake of iron. Siderophore production by the strain at 39°C could not be studied since the strain failed to grow at this temperature. However, growth with addition of siderophore at 39°C has not been studied. It is possible that siderophore production or any other biosynthetic pathway is affected at 39°C and above.

Ismail et al. (1985) working on Candida albicans, showed that elevated temperatures did not inhibit siderophore production of the organism rather only a decrease in the rate and quantity of siderophore production were apparent. The optimum growth temperature of Candida is considered to be around 25°C, but the effect of high temperature on the growth and siderophore production was assessed by growing the organism at 37°C and 41°C. Regardless of the incubation temperature or iron-limitation of the culture media, the organism secreted approximately equal amounts of siderophore for the first 8 days of incubation. Thereafter, a significant increase in the quantity of siderophore production occurred at 37°C in the deferrated medium as compared to the quantity produced at 41°C. Elevated temperature did not affect the amount of siderophore secreted in the control medium. In this organism elevated temperature also did not affect the growth of cells in control medium. But a significant suppression of growth compared to the control was observed in the deferrated medium at both the temperatures. However with time, the growth rate of candidal cells in the deferrated medium showed partial
recovery when compared to the growth rate in the control medium. This recovery in growth was followed by an increase in siderophore production at both temperatures. Although greater quantities of siderophore were produced at 37°C in the deferrated medium, no significant difference was apparent in the growth at 37°C and 41°C. Possibly this result occurs because both media contain a fixed quantity of iron and regardless of the quantity of the siderophore no additional supplementation of growth occurs.

Neilands (1984) reported that siderophore production is affected by carbon source in the growth medium. *Rhizobium* strain BICC 651 is capable of growth in a large number of carbon sources including sugars, sugar alcohols and organic acids. Glucose, while generally a preferred carbon source, does not allow high siderophore production by strain BICC 651. Growth on succinate and pyruvate, on the other hand, gives higher yields (Table 10). Apparently, this is because succinate and pyruvate are metabolised through the tricarboxylic acid cycle which requires an iron-containing system. As a consequence, the cells rapidly become depleted in iron resulting in higher production of the siderophore. Malate is a product of both arabinose and succinate metabolism and upto 3 mM is produced during the late exponential or early stationary phase by all root nodule bacteria growing on arabinose and succinate (Carson et al, 1992 a). Malate, a C₄ dicarboxylate, appeared to have no relationship with external iron concentration and did not support DHBA production by strain BICC 651.
The results described in this work show that *Rhizobium* strain BICC 651 produced a siderophore which was identified as 2,3-DHBA. This is the first report of release of 2,3-DHBA under iron deficiency by a strain of *Rhizobium* isolated from *C. arietinum*, although strains of *R. leguminosarum* bv. *trifolii* (Skorupska *et al*, 1988) and *R. leguminosarum* bv. *viciae* (Patel *et al*, 1988) were reported to produce 2,3-DHBA. Chemical assay indicated production of anthranilic acid, a siderophore structurally related to 2,3-DHBA from a strain of *R. leguminosarum* bv. *viciae* (Rioux *et al*, 1986 a), although some doubt was expressed by Rosendahl *et al* (1991) about the reports of catechol siderophore production by *Rhizobium*. We identified the siderophore of BICC 651 as 2,3-DHBA by chromatographic comparison with reference compounds (Table 11), analyses of mass spectrum (Fig.10) and UV- (Fig. 11) and IR spectra (Fig. 12) comparison with an authentic sample. In an initial experiment, upon TLC, the siderophore of BICC 651 was found to yield a single spot with an R_F value similar to that of authentic 2,3-DHBA. A mixture of 2,3-DHBA and of siderophore of strain BICC 651 upon co-chromatography also yielded a single spot with R_F value similar to that of 2,3-DHBA. The core compound of the siderophore of BICC 651 was then isolated and subjected to analyses. The curve was superimposable to that of authentic 2,3-DHBA. The mass spectrum of the core compound of the siderophore of BICC 651 showed a molecular ion peak at M^+ 154. The other significant peaks were at m/z 136, 108 and 80. The peak m/z 136 obtained from the molecular ion peak (M^+ 154) indicated loss of mass 18 from the molecular
peak which is characteristic of O-hydroxybenzoic acid (Silverstein et al., 1981). UV spectra of both the core compound of the siderophore of BICC 651 and authentic 2,3-DHBA showed three peaks with $\lambda_{\text{max}}$ in ethanol at 315 nm, 250 nm and 210 nm respectively and the spectra were superimposable. The absorption maxima at 210 and 315 nm of 2,3-DHBA were due to presence of benzene ring and a,b-unsaturated ketone (C=C-C) respectively. The IR spectrum of the core compound of the siderophore of BICC 651 showed absorption bands at $\nu_{\text{KBr}}$ max 3500, 1720 and 1600 cm$^{-1}$ suggesting presence of hydroxyl group, acid carbonyl group and aromatic system respectively.

Acid hydrolysis of siderophore followed by paper chromatographic comparison of the hydrolysate with authentic samples led us to conclude that the siderophore contains threonine as its amino acid conjugate (Table 12). Analysis of siderophore with HPLC with an inbuilt system of hydrolysis and separation of amino acids in its column strengthened the observations of paper chromatography that threonine was indeed the amino acid that remained conjugated to siderophore of BICC 651 (Fig. 13). From the peak area the quantity of threonine present in a given amount of siderophore (measured by Arnow reaction) was estimated and the molecular ratio of the amino acid conjugate to DHBA was estimated to be 1.6:1. This value is in close approximation of 2:1, indicating two threonine moieties, possibly as threonyl-threonine are linked to the -COOH group of the core compound, DHBA by peptide bond. Threonine was previously detected in siderophore of R. leguminosarum bv. viciae

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by Patel et al (1988). Also, production of a catechol-like (2,3-DHBA) siderophore containing glycine and threonine by cowpea Rhizobium RA-1 was detected by Modi et al (1985).

The study of growth inhibition of the strain BICC 651 by the addition of synthetic iron chelator EDDA to a final concentration of 4 mg ml$^{-1}$ in Complete medium at various time points show that the greatest inhibition of growth of the strain was achieved when EDDA was added 24 h prior to inoculation (Fig. 14). EDDA added to the culture at the time of inoculation did not cause any extended lag although there was considerable growth inhibition. When EDDA in the same concentration was added to the culture of BICC 651 after 10 h of growth and the culture reached an OD$_{590}$ of 0.5, the growth inhibition effect was less pronounced compared to the culture where EDDA was added 24 h prior to inoculation. The data are in conformity with the fact that the earlier the EDDA is added, the better is the chelation of iron and the better the chelation of iron the higher the growth inhibition. As such, a 24 h chelation period was allowed in all the subsequent experiments to study EDDA-imposed growth inhibition.

When EDDA at varying concentrations were used for chelation of iron for 24 h, an EDDA concentration dependent inhibition of bacterial growth was noticed (Fig. 15). While the growth of the strain BICC 651 was totally inhibited by 4 mg ml$^{-1}$ EDDA added to the Complete medium, in case of the strain BICC 632 only, 2 mg ml$^{-1}$ EDDA was needed to inhibit the growth completely. Bradyrhizobium sp. (Cicer arietinum) TAL 1012, however, being a slow grower needed only 0.1 mg EDDA ml$^{-1}$ of medium. From the
result it is apparent that the strain BICC 632 was almost two-fold more sensitive to EDDA as compared to the strain BICC 651. The strain *Bradyrhizobium* sp. TAL 1012 was highly sensitive to the presence of EDDA in the medium.

In order to examine if growth inhibition by the added iron chelator EDDA is a reversible process, the effect of addition of FeCl₃ at different concentrations to the EDDA added culture was studied. It appeared from the result (Fig.16) that with addition of 0.8 mmol ml⁻¹ or 1.2 mmol ml⁻¹ FeCl₃ in 4 mg ml⁻¹ EDDA added medium the extent of growth of the strain BICC 651 improved considerably. However, even at 1.2 mmol ml⁻¹ FeCl₃ could not completely overcome the effect of 4 mg EDDA ml⁻¹ medium as was evident from the growth rate and the extent of growth of BICC 651. It appears that even higher concentration of FeCl₃ is necessary to compensate for the effect of the added EDDA. Buyer & Leong (1986) showed that siderophore pseudobactin A214 from bean-deleterious *Pseudomonas* A214, and ferrispseudobactin A214, both at 10 μM, were about equally effective in reversing iron starvation of the *Pseudomonas* strain A214 induced by the synthetic ferric complexing agent EDDA in the plate bioassay. This stimulation of growth was evidenced by growth of cells surrounding the paper discs containing the above compounds. In contrast, FeCl₃.6H₂O at 10 mM was apparently required to saturate the EDDA in the medium, thereby producing similar-sized zones of growth as the above compounds. Hence it is evident that *Pseudomonas* A214 could utilise pseudobactin A214 to transport Fe³⁺ and an one thousand fold iron is needed to compensate for the siderophore.
To assess if the siderophore isolated from iron-limited culture of strain BICC 651 was active in iron transport and as such could provide necessary iron to the organism itself in an iron-poor environment, the reversal of EDDA imposed growth inhibition of the strain by added siderophore was examined. When purified siderophore from strain BICC 651 (8.2 μ mol equivalent of DHBA ml⁻¹) was added to the EDDA-inhibited culture at 40 h, growth of the bacteria resumed with a generation time comparable to that of the control culture with equivalent cell yield (Fig. 17).

So, it was evident that the siderophore isolated from the iron-limited culture of BICC 651 can support iron nutrition of the parent strain, thus demonstrating its biological property.

To investigate if 2,3-DHBA, which constitutes the core of the siderophore of strain BICC 651, could itself serve to transport iron to the strain, the compound was added to the EDDA-inhibited culture at 40 h at different concentrations. The result showed that 2,3-DHBA was capable of alleviating growth inhibition of BICC 651 imposed by EDDA, supplying much needed iron for the growth of the strain (Fig.18). It was also apparent from the result that 83 μ mol 2,3-DHBA ml⁻¹ of culture could provide the necessary iron to achieve the extent of growth and growth rate similar to that provided by the siderophore of the strain with 8.2 μ mol equivalent of DHBA ml⁻¹. As such, DHBA seems to be about 10 times less effective than the siderophore of BICC 651 in transporting iron.
Since Rhizobium sp. (Cicer arietinum) BICC 632 also produced a catechol type of siderophore and the siderophore produced by it yielded a CAS positive spot having similar R<sub>F</sub> value (0.82) to that of BICC 651 (0.80) (Table 8), it was interesting to investigate if the siderophore produced by BICC 651 could serve as an agent of iron transport also for the strain BICC 632 and would allow the strain BICC 632 to overcome the EDDA-imposed growth inhibition and in attainment of its normal growth. In a similar experiment, if siderophore produced by strain BICC 651 could serve as an iron-carrier for Bradyrhizobium sp. (Cicer arietinum) TAL 1012, which did not produce any siderophore in Complete medium, was examined by reversal of EDDA imposed growth inhibition. In both cases the siderophore from BICC 651 was found to reverse the EDDA-imposed growth inhibition of the bacterial strains BICC 632 and TAL 1012 (Figs. 19 & 20). The present investigation provides evidence that the strains BICC 632 and TAL 1012 both isolated from Cicer arietinum could utilise the siderophore produced by Rhizobium BICC 651 which also originated from Cicer arietinum. However, TAL 1012 is a slow-growing strain and belongs to the genus Bradyrhizobium and yet the strain is capable of utilising the siderophore produced by a fast-growing Rhizobium. Thus it appears that cross feeding of siderophore is not limited by taxonomic boundary of the organisms. Utilisation of the same siderophore by different strains suggests that interstrain synergism may occur in the rhizosphere. On the other hand, antagonism among strains through the production of siderophore is also likely to occur in the rhizosphere.
Siderophore production and utilisation of ferric chelate of siderophores coupled with growth inhibition of other organisms by depriving them of iron following its chelation by the siderophore of the producer organism should play a key role in competition (Reigh and O'Connell, 1993).

Strain-specific utilisation of siderophores in a *Rhizobium* species was first reported by Smith and Neilands (1984) who showed that *R. meliloti* DM4 excreted and utilised the siderophore rhizobactin, whereas several other wild-type *R. meliloti* strains did not. Siderophore-mediated competition for iron in microbial systems appears to be a widespread phenomenon. *Pseudomonas fluorescens* residing in the rhizosphere of plants secretes the siderophore pseudobactin (Teintze et al, 1981) profusely, as a result all the available iron surrounding the root of the plant become bound to pseudobactin. Since the pseudobactin-iron complex can only be utilised by *P. fluorescens* which has the appropriate receptor for it, all other microorganisms in the rhizosphere including the pathogenic fungi are starved of iron and their growth is inhibited. In the rhizosphere, similar to *Pseudomonas*, a *Rhizobium* strain producing siderophore could outcompete other strains of *Rhizobium* or any other microorganism requiring iron but producing no siderophore (Kloepper et al, 1980). However, a *Rhizobium* strain capable of utilising a siderophore produced by another strain of *Rhizobium* or any other organism should also have a competitive advantage in the rhizosphere, even if it does not produce any siderophore itself. Smith & Neilands (1984) reported that *R. meliloti* strain DM4 utilised not only its own
siderophore, rhizobactin, but also ferrichrome and ferrioxamine B, although, the bacterium could not utilise mugeneic acid, pseudobactin, citric acid, enterobactin or aerobactin. Skorupska et al (1989) reported that 7 out of 8 strains of R. leguminosarum bv. trifolii screened for siderophore utilisation could not use pseudobactin, citrate or desferal. The lone strain remaining produced siderophore, and also grew better in the presence of all the three siderophores, i.e., pseudobactin, citrate and desferal, but whether it was utilising the siderophore directly was not assessed. Rioux et al (1986 a, b) also reported the utilisation of anthranilic acid and citric acid by a strain of R. leguminosarum bv. viciae based on both growth stimulation and uptake studies.

The optimum pH of ferrisiderophore uptake by iron-deficient cells of BICC 651 was pH 7.0 (Fig. 21) coinciding with the optimum pH of growth. However, the optimum temperature of ferrisiderophore uptake was found to be 35°C (Fig. 22) and ferrisiderophore uptake occurred at temperature even beyond 40°C. Thus, the total inhibition of growth of the strain BICC 651 at 39°C as observed earlier is possibly not due to impaired uptake mechanism.

The transport of ferrisiderophore in Rhizobium strain BICC 651 (Fig.23) appears to be similar to that of R. leguminosarum (Rioux et al , 1986 b). During transport experiments more than 90% of added iron was still available for uptake by cells after 30 min. In E. coli transport of different ferrisiderophores requires different receptors in the outer membrane and also
functional Ton B and Exb B proteins (Pressler et al, 1988). All these genes are controlled at the level of transcription by the Fur (Ferric uptake repressor) protein. Under low-iron conditions, repression by Fur is relieved and the genes are transcribed. Under conditions of iron sufficiency, Fur negatively represses the transcription of all these genes (Bagg & Neilands, 1987a; de Lorenzo et al. 1987; Griggs & Konisky, 1989). Transport in strain BICC 651 may also require products of similar genes since cells grown in 50 μM FeCl₃ transport very little ⁵⁹Fe³⁺. In fact, not only transport of iron but also synthesis of siderophore is affected severely in iron sufficient condition and both siderophores and receptors are not synthesised at this condition. In the absence of siderophore, transport of free ⁵⁹ Fe³⁺ is also low. These data indicate that the high affinity transport is inducible and siderophore-mediated as is observed in E. coli and Pseudomonas aeruginosa (Frost and Rosenberg, 1973; Cox 1980b). With mannitol-grown cells the uptake of iron appeared to be biphasic in contrast to succinate-grown cells (Fig. 23, inset). There was an initial rapid uptake followed by a slower rate of transport. Similar biphasic nature of ferric citrate uptake was seen in Bradyrhizobium japonicum by Guerinot et al. (1990) when the cells were grown without iron. Using radiolabeled (⁵⁵Fe) ferric citrate it was shown that there was an apparent uptake for the first two min followed by a slower, linear uptake over the next 25 min. Azotobacter Vinelandii has also been reported to have a biphasic uptake of its own siderophores (Knosp et al., 1984). The rate of uptake of iron in both mannitol and
succinate-grown cells appeared to be similar either in presence or absence of a carbon source in uptake medium. It is not quite certain if the uptake is an active process in BICC 651. To determine this it will necessitate studying iron uptake by the cells in the presence of energy inhibitors (Carson et al, 1992 a). The reason why the uptake is biphasic in mannitol-grown cells in contrast to the monophasic nature of uptake in succinate-grown cells has not been made clear in this study.

The uptake of $^{59}\text{Fe}^{3+}$ in the form of ferric-siderophore complex was found to be dependent upon the substrate concentration (Fig. 24). The maximum rate of uptake was found to be more than 2 n mol (ml cells)$^{-1}$ 90 min$^{-1}$. The $K_m$ value of iron uptake was calculated to be 0.57 µM; this indicates the high affinity of the receptor protein in the outer membrane of the strain BICC 651 towards the siderophore-iron complex. That the utilisation of siderophore was due to the presence of a specific OMP was demonstrated by comparing the OMP profiles of Rhizobium strain BICC 651 grown in the presence or absence of iron. Two peptide bands of molecular mass 82 kDa and 76 kDa were found to be expressed only under iron-deficient conditions in the OMP (Fig. 25 a, b). Although specific experiments were not carried out to test the receptor functions of these proteins, it is taken a priori that these two peptides could act as the receptor proteins for recognition of ferrisiderophore. Strong evidence of a receptor nature for such proteins was provided by the studies of Reigh and O’Connell (1993) who compared the OMP profiles of R. meliloti 102F34::pGR 30 with the profiles of R. meliloti strains
2011, 220-5 and 220-3. The strain 102F34 ::pGR 30 produced a siderophore and could utilise the siderophores of the latter strains, whereas the parent strain *R. meliloti* 102F34 did not produce a siderophore and could not utilise the siderophores of *R. meliloti* strains 2011, 220-5 and 220-3. In *R. meliloti* 102F34::pGR 30 a 72 kDa protein, which was not present in *R. meliloti* 102F34, was expressed only under low-iron conditions. The 72 kDa protein was also induced in *R. meliloti* 2011, 220-5 and 220-3 under low iron conditions. Thus, in these strains 72 kDa protein was the only receptor protein of the ferrisiderophore transport system.

The release of iron from its carrier in many microorganisms is mediated by a ferrireductase. The enzyme reduces Fe$^{3+}$ of ferrisiderophore to Fe$^{2+}$ which is then released. A ferrireductase was identified in soluble cell extract of *Rhizobium* strain BICC 651. This finding confirmed that ferrisiderophore was transported across the bacterial membrane before removal of iron. The enzyme was active even under aerobic conditions as is the ferrireductase from *Azotobacter vinelandii* (Huyer & Page, 1989). However, like most ferrireductases from other microorganisms (Arceneaux, 1983), the creation of a partially anaerobic environment resulted in increased activity. This was also accompanied by an increased level of non-enzymic reduction of iron and the levels of enzymic activity varied widely. Since we were unable to resolve these problems, we therefore, used conditions that were not strictly anaerobic. The activity of the ferrireductase from strain BICC 651 was not
affected by the level of iron present in the growth medium; therefore, the enzyme appeared to be constitutive, unlike ferrireductase of certain fungi (Ernst & Winkelmann, 1977).

The reaction of the enzyme was stimulated in presence of magnesium while addition of other cations produced stimulation or was inhibitory (Table 13) as was also observed with the ferrireductase from Agrobacterium tumefaciens for the reduction of ferri-DHBA (Lodge et al., 1982). Mg\(^{2+}\) could be effectively replaced with Ca\(^{2+}\) which was also a divalent cation. Relative to activity in the absence of Mg\(^{2+}\), other metal ions except Al\(^{3+}\), were inhibitory to the activity.

The properties of the enzyme were similar to those recorded for other ferrireductases in that the enzyme required FMN as a cofactor and utilised either NADH or NADPH as a reductant. However, succinate, normally a good reductant for membrane bound reductases, was not a good reductant for the reductase in Rhizobium strain BICC 651 (Table 14).

From the activity staining in native gel two forms of the enzyme ferrireductase were apparent, as was also found in Rhodopseudomonas sphaeroides (Moody & Dailey, 1985) and in Pseudomonas aeruginosa (Cox, 1980a).

Using different concentrations of DHBA with a fixed amount of FeCl\(_3\), where DHBA:FeCl\(_3\) molar ratios varied from 0:1 to 6:1 in the incubation mixture, to allow complex formation and then using the incubation products as substrate in enzyme assays the highest activity of the enzyme occurred when FeCl\(_3\) was complexed with

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atleast three-fold higher DHBA concentration. The data implied that most probable stoichiometry of the complex was 1 mole of Fe$^{3+}$ with 3 moles of DHBA (Fig. 26, inset). As such, in further experiments involving ferrireductase activity the substrate used was a complex of FeCl$_3$ and DHBA mixed in the ratio of 1:3.

Using different concentrations of Fe$^{3+}$-DHBA (FeCl$_3$:DHBA:: 1:3) complex as the substrate, where concentration refers to the concentration of iron, it was revealed that the semipurified enzyme, ferrireductase, from strain BICC 651 had an apparent $K_m$ of 0.3 mM (Fig. 26). When only FeCl$_3$ was used as the substrate the $K_m$ of the enzyme was determined to be 0.33 mM (Fig. 27). Though the $K_m$ values of the enzyme were very much comparable for the two substrates, the $V_{max}$ value of the enzyme was 1.4 times higher when FeCl$_3$ was used as the substrate than with Fe$^{3+}$-DHBA complex. The $K_m$ values observed, appeared to be high considering that the $K_m$ values of enzymes of related organisms such as from Pseudomonas aeruginosa was 56 $\mu$M for ferripyochelin, although it was 303 $\mu$M for ferric citrate and the organism had two enzymes for the two substrates (Cox, 1980 a), from Azotobacter vinelandii $K_m$ was 10 $\mu$M for ferric citrate (Huyer & Page, 1989), and it was 8.3 $\mu$M also for ferric citrate from Rhodopseudomonas sphaeroides. However, the $K_m$ value for the reductase for ferrischizokinen, the ferrisiderophore produced by Bacillus megaterium, a Gram-positive bacterium, was as high as 250 $\mu$M (Arceneaux & Byers, 1980). Of the two enzymes in P. aeruginosa, oxygen irreversibly inactivated the ferripyochelin iron reductase whereas the ferric citrate iron reductase with a high $K_m$ was
essentially unaffected by oxygen. The susceptibility of the two forms of ferrireductase observed in Rhizobium strain BICC 651 towards oxygen is not known. The high $K_m$ value of the ferrireductase of BICC 651 indicates low affinity of the enzyme towards the substrate ferri-DHBA. It is highly possible that the two isoforms found in BICC 651 have different affinities towards the substrate ferri-DHBA. If the one with high affinity towards ferri-DHBA is $O_2$ sensitive and loses its activity upon exposure to $O_2$ and while the other with low affinity towards ferri-DHBA is not affected by $O_2$, under such circumstances a high $K_m$ is likely to be apparent. Thus the high $K_m$ of the enzyme for ferri-DHBA may be an indication of oxygen sensitivity of a form of the enzyme.

The fact that the enzyme ferrireductase from strain BICC 651 uses ferrisiderophore as its substrate is indicative that the enzyme is used for iron assimilation. However, it would require isolation and studies of mutants of ferrisiderophore reductase of the strain for an evidence that it is essential in vivo for iron transport by the siderophore system and that its function is to release the iron of ferrisiderophore for cell metabolism.

Although siderophores are produced in response to iron limitation, there are other metal ions found in the natural environment of microorganisms which could potentially have an effect on iron assimilation or could interact with the iron binding ligand. As such, it was interesting to examine if different metal ions present in the natural habitat of the
bacteria other than iron would have any effect on the production of siderophore of the strain BICC 651.

A highly striking role of Al$^{3+}$ influencing growth and siderophore production of *Rhizobium* sp. (*Cicer arietinum*) BICC 651 was revealed. The extent of growth of the strain BICC 651 decreased linearly with increasing concentrations of Al$^{3+}$, if only Fe$^{3+}$ was limited in the medium. The data (Figs. 28-30) indicate that aluminium is toxic to the organism. The inhibitory growth effect of Al$^{3+}$ could be alleviated by a simultaneous addition of Fe$^{3+}$ (Fig. 31) which indicated a probable antagonism between Fe$^{3+}$ and Al$^{3+}$. The fact that in presence of 100 $\mu$M Fe$^{3+}$, addition of even 1 mM Al$^{3+}$ did not result in significant change in the growth pattern of the strain attest to this view. Also, it appears that addition of Fe$^{3+}$ (about 10 $\mu$M) could alleviate the effect of 100 $\mu$M Al$^{3+}$ on the growth of the organism. It is to be noted that a 2.4-fold increased level of siderophore was recorded in presence of 100 $\mu$M Al$^{3+}$ (Fig. 28-30) in an iron-deficient medium. Taking all the data into consideration it is rationalised that the siderophore produced in an iron-limited medium becomes complexed with added Al$^{3+}$ leaving little free siderophore for iron uptake. This results in significantly reduced or no iron transport at all creating iron deficiency inside the bacterial cells. This caused further induction of siderophore synthesis to get increased access to iron and to transport it from the medium to inside the cells. If this is true, it would mean that Al$^{3+}$ binds to the siderophore in a manner similar to that of Fe$^{3+}$. This, should result in a competition between Al$^{3+}$ and Fe$^{3+}$ for
binding to siderophore. In that case, however, Al\textsuperscript{3+} would prove to have a low affinity for the siderophore since 10-100 fold less concentrations of Fe\textsuperscript{3+} was found to alleviate the effect of Al\textsuperscript{3+} on growth of the organism (Fig. 31). As a matter of fact, in iron-sufficient condition (100 \textmu M), Al\textsuperscript{3+} even at 1 mM concentration did not result in significant growth inhibition of the strain (Fig. 33). Also, in presence or absence of 100 \textmu M Al\textsuperscript{3+} siderophore production was practically reduced to zero with 100 \textmu M added FeCl\textsubscript{3} (Fig. 32).

The purified siderophore from Al\textsuperscript{3+}-supplemented iron-deficient culture was characterised also to be 2, 3-DHBA with threonine as conjugate, as expected, in view of the fact that it is the deficiency of iron and not Al\textsuperscript{3+} per se triggers increased synthesis of siderophore. The protein profile of the OM remained unchanged during growth in 100 \textmu M Al\textsuperscript{3+} as against the control (Fig. 34). This indicated that Al\textsuperscript{3+} does not affect the receptor profile and iron acquisition system to a large extent. However, in the present study it is not clear whether Al\textsuperscript{3+} after binding to siderophore of the strain BICC 651 is transported inside the cells or not. Nevertheless, Al\textsuperscript{3+} toxicity that results in decreased growth of the strain in absence of added iron points towards entry of Al\textsuperscript{3+} inside the cell. Since Al\textsuperscript{3+} does not have a divalent state, it then can not be subjected to siderophore reductase activity to separate Al\textsuperscript{3+} from Al\textsuperscript{3+}-siderophore complex. However, it is not known if any other mechanism such as an esterase activity could cause release of Al\textsuperscript{3+} from its siderophore complex. The other explanation of Al\textsuperscript{3+} toxicity could
be due to competition of Al$^{3+}$ with Fe$^{3+}$ and under Fe$^{3+}$ limited condition presence of Al$^{3+}$ in the medium engages the siderophore by being bound to the Fe$^{3+}$ binding sites, thus reducing the transport of limited iron even further. This would cause severe iron deficiency in the cells, thereby resulting in decreased growth.

The effects of zinc and molybdenum on growth and siderophore production by the strain *Rhizobium* sp. BICC 651 were also studied. Biomass yield of the strain BICC 651 was affected only slightly in the presence of Zn$^{2+}$ as high as 1 mM. The level of siderophore was, however, found to decrease with an increase in the concentration of ZnSO$_4$ (Fig. 35). Huyer & Page (1988) observed that an addition of 20 or 40 μM ZnSO$_4$ caused an increased production of azotobactin, the siderophore of *Azotobacter vinelandii*. Two possible reasons might be thought of to accommodate the enhancement of siderophore production by ZnSO$_4$. That ZnSO$_4$ may play a role of cofactor of any one of the biosynthetic enzymes needed for siderophore production pathway; the other possible explanation for the observed increased production of siderophore during growth in the presence of Zn$^{2+}$ being that the zinc ions were binding competitively with iron to the siderophores, necessitating increased siderophore production to obtain equivalent level of needed iron (Huyer & Page, 1988).

The authors also observed that Zn$^{2+}$ did not affect production of the high-molecular weight outer membrane iron-repressible proteins that presumably function as ferrisiderophore receptors. On the contrary, the authors found that cultures grown in the
presence of Zn$^{2+}$ appeared to produce greater amounts of these high molecular weight iron-repressible proteins (93, 85, 81 and 77 kDa), a result consistent with the concomitant increase in siderophore production.

In 1984 Hider hypothesised the iron acquisition systems to be involved also in the transport of other essential transition metals required for the activity of metalloenzymes containing molybdenum, cobalt, nickel and copper. Pope et al (1980) documented the chelation of molybdate by catechol groups. Extracellular siderophore, N,N-bis (2,3-dihydroxybenzoyl)-L-lysine, produced by Azotobacter vinelandii in response to iron deprivation appeared to bind molybdate (Page & von Tigerstrom, 1982). The dependence of nitrogen fixation on molybdenum has been known for a long time (Bortels, 1930). This prompted us to investigate the effect of this metal ion on growth and siderophore production of Rhizobium BICC 651, a nitrogen fixing symbiotic organism.

The biomass yield of the strain remained unaffected by the addition of molybdenum upto a concentration of 100 $\mu$M. Further addition of molybdenum, however, resulted in decreased cell yield revealing toxic effect of molybdenum on the growth of BICC 651. However, with 400 $\mu$M added molybdenum, siderophore production was found to be optimum and the level was 1.4 times higher than that recorded in the control culture (Fig. 36). This is possibly due to the binding of molybdenum to the siderophore and thus making iron unavailable to the organism. So an increased production of siderophore is needed to chelate iron from the medium and supply
Page & von Tigerstrom (1982) have reported the hyperproduction of a 44 kDa protein and production of a minor 77 kDa protein in the outer membrane of nitrogen fixing cells of *Azotobacter vinelandii* under Mo limitation. This result also offers substantial evidence that Mo may be transported in *Azotobacter vinelandii* by a siderophore-mediated system. Saxena et al (1989) showed the secretion of 3,5-DHBA by *Azospirillum lipoferum* D-2 in the growth medium when the cells became limited in either molybdenum or both. When both iron and molybdenum were supplemented in the growth medium, production of 3,5-DHBA was repressed. In presence of iron production of 3,5-DHBA occurred only under Mo-limited condition. In absence of iron, however, 3,5-DHBA was produced irrespective of presence or absence of Mo. Under such a condition, the production was higher in absence of Mo. The authors showed that the presence of 3,5-DHBA in the uptake system resulted in facilitated uptake of Mo by *A. lipoferum* D-2 suggesting the role of 3,5-DHBA, as a Mo-transporting compound in this organism. SDS-PAGE of the outer membrane of *A. lipoferum* D-2 revealed the appearance of a new protein of 78 kDa and the overproduction of a 88 kDa protein which was otherwise present in the Mo-grown cells at constitutive level. The work of Saxena et al (1989) provided substantial evidence that Mo may be transported by *A. lipoferum* D-2 by a siderophore-mediated system. Whether this system is constitutive or is induced under Mo-limited nitrogen-fixing conditions is not known. Facilitated transport of iron (Saxena et al, 1986) as well as molybdenum in the presence of 3,5-DHBA might be the result of
a common transport system that the organism has evolved to satisfy the requirement for these metals, since iron as well as Mo forms an integral component of nitrogenase.

**Rhizobium** strain BICC 651 is an effective nitrogen-fixing one and it profusely nodulates its host which may be due to a selective advantage conferred by its siderophore production in the rhizosphere. To examine this possibility several mutants in siderophore production were isolated and characterised. From the comparison of nodulation efficiency of the wild type strain BICC 651 and its siderophore over-producing and siderophore non-producing mutants, it is evident that siderophore over-producing mutant (N15) is more efficient in respect of inducing total number of nodules on its host than its wild type counterpart (BICC 651) or the siderophore non-producing mutant (A35) (Table 17). It seems that there might be a correlation with the siderophore producing capability of the **Rhizobium sp.** (*Cicer arietinum*) BICC 651 with the nodule number. It has been suggested that differences in nodule development under iron-deficient conditions may be due to varying abilities of different strains of root-nodule bacteria to acquire iron for nodule initiation and development (O'Hara *et al*, 1988). Siderophore over-producing mutants may be better suited for iron acquisition and nodule initiation. Barton *et al* (1992) examining the wild type *R. meliloti* 1021 and its Tn5 mutants with altered rhizobactin activities observed that the greatest level of growth was with the wild type and although there was no strict correlation between the nodule number per plant and rhizobactin production,
in general, less number of nodules were produced by siderophore negative mutants. These authors did not raise any siderophore over-producing mutants, however. Nevertheless, a correlation was observed between the amount of nitrogen fixed per bacteroid in the nodule and the siderophore producing ability.

Manjanatha et al (1992) evaluated Tn5 mutants of R. fredii overproducing siderophores for their competitive abilities in an alkaline soil. The Tn5-carrying mutants when further tested in the greenhouse for their competitiveness against native strains present in an alkaline soil of Iowa and for their symbiotic activity, it was observed that nodule occupancies in the greenhouse by the two siderophore overproducing mutants of R. fredii were 3 and 4%, compared with 19% for the wild type strain. The authors interpreted that either wild type was acquiring all the iron needed for maximum growth and nodulation, or the insertion of Tn5 and the overproduction of siderophore resulted in less competitive strains. The study however, did not map the site of integration of Tn5 sequence in the organism's DNA. The polar mutation caused by Tn5 insertion should theoretically cause inhibition of transcription of the downstream genes to the inserted sequence. The overproduction of siderophore would imply that either Tn5 insertion took place in the regulatory gene or the genes for the transport proteins. If the transport genes were affected, the transport proteins would not be transcribed and iron transport would be restricted. This would result in iron deficiency within the cell triggering further siderophore synthesis. However, the overproducing strains being limited in
their iron transport capability most probably will be restricted in their nodulation property as also was seen by the authors. The high siderophore producing mutants isolated by us do not appear to be affected in the regulatory gene of siderophore production since Fig. 41 shows in all the cases 50 µM iron represses siderophore biosynthesis in all of them and the strain N15 produces 1.3 times high nodule number in its host. It is possible that during NTG mutagenesis some other regulatory gene apart from Fur gets affected causing higher transcription of genes responsible for siderophore synthesis. Another interesting observation came from the work of Derylo & Skorupska (1992) who examined the effect of rhizobial siderophore on the symbiotic properties of nodulating and nitrogen fixing (Nod+ Fix+) R. leguminosarum bv. trifolii 24. They showed that the catechol siderophore isolated from supernatant of R. trifolii AR6- a non nodulating mutant of R. leguminosarum bv. trifolii 24 reduced considerably the nitrogenase activity of clover plant. Although higher concentrations of the siderophore were harmful both to nodulating and non-nodulating clover plant, the nodulating plants were especially sensitive to the toxic effect of the rhizobial siderophore. This was evident from the fact that the chelator at a concentration of 5 µM did not reduce the production of chlorophyll a and b (1.07 and 0.29 mg g⁻¹ fresh matter respectively) in the non-infected clover plant compared to that where no siderophore is added (0.88 and 0.38 mg g⁻¹ fresh matter). However, the concentration of siderophore higher than 5 µM decreased both chlorophyll contents and fresh weight of the clover plants. This results indicate that the toxic effect of the
rhizobial siderophore was not caused by the inhibition of iron utilisation, because normal or even enhanced synthesis of chlorophyll was observed when the clover plant was treated with siderophore. The toxicity of the rhizobial siderophore can be explained by the greater availability of the iron and its excess is harmful. The excessive concentration of this cation caused generation of the oxidizing radicals. The authors, however, thought of another possibility that the siderophore disturbs the transport of other cations.