Chapter 1

Review of Literature

Our understanding of the world is built on innumerable layers. Each layer is worth exploring, as long as we do not forget that it is one of many. Knowing all there is to know about one layer—a most unlikely event—would not teach us about the rest.

Erwin Chargaff
Chapter 1: Review of Literature

1.1 IRON METABOLISM IN BACTERIA

Iron is involved in various biological activities of microorganisms including many that are vital in a normal cellular metabolism (Byers and Arceneaux, 1977). Barring few species such as *Streptococcus sanguis*, some *Lactobacillus* species, and *Borrelia burgdorferi* (Guiseppe and Fridovich 1982, Archibald 1983, Posey and Gherardini 2000), iron is an essential requirement for growth of bacteria and its deficiency leads to partial or complete inhibition of the growth. Iron status also influences virulence of some pathogens since in animal tissues, iron is complexed to various proteins e.g. transferrin, haemoglobin, lactoferrin, ferridoxin, flavoprotein, ferritin, iron-sulfur proteins etc. (Bullen, 1981). Iron participates in many biological processes, including electron transport chains, and is a cofactor of enzymes of intermediary metabolism likeaconitase, peroxidases and catalases, ribonucleotide reductase. The existence of two oxidation states of iron, ferrous (Fe$^{2+}$) and ferric (Fe$^{3+}$), within the physiological redox range gives iron its ability to act as an electron transporter (Bullen, 1981; Neilands, 1982; Neilands, 1984; Crosa, 1989). Both Fe$^{2+}$ and Fe$^{3+}$ are relatively small ions and have a ability to form six-coordinate complexes with ligands containing O, N and S and this property plays a critical role in making iron participate in fundamental reactions of oxygen metabolism, electron transfer, RNA synthesis and other processes (Archibald, 1983; Braun, 1997; Crosa, 1997).

1.1.1 Biogeochemistry of iron

It may be assumed that iron containing enzymes played a significant role in internal redox reactions during the Earth’s anaerobic phase of life. At that time no special transport system was needed then to transport the abundant ferrous ions, since this oxidation state is highly soluble even at physiological pH. But gradually as the atmosphere turned aerobic due to the evolution of O$_2$ as a result of oxygenic photosynthesis, it precipitated the surface iron of Earth as an oxyhydroxide polymer. In aqueous solutions and at neutral pH, this insoluble polymer [Fe(OH)$_3$] has a solubility constant of $\sim 10^{-38}$ M, so very little is available as soluble Fe$^{3+}$. At pH 7.0, the solubility of free iron is therefore $10^{-17}$ M (Bullen, 1981; Neilands, 1982; Neilands, 1984), a concentration too low to allow growth of aerobic...
Nevertheless, the requirement for iron is almost universal for prokaryotes and eukaryotes demonstrating its importance in early cellular metabolism.

1.1.2 Iron uptake mechanisms in bacteria

Bacteria have evolved a range of strategies to acquire iron. Under anaerobic conditions, Fe$^{2+}$ is sufficiently soluble, for anaerobic bacteria to transport it. Under aerobic conditions there are different ways to do so. Experiments with a number of bacteria using mutational analysis have shown that there are multiple ways in which iron is transported into a cell system. These may be differentiated into two main iron uptake systems depending upon the absence and presence of an iron chelator respectively as low affinity and high affinity (Neilands, 1984).

1.1.2.1 Low affinity iron-uptake system

It was found that the mutant of *Salmonella typhimurium* LT2 defective in the synthesis of an iron chelator (enterobactin) was still able to grow at normal rates in nutrient broth (Pollack et al., 1970). This showed the presence of a low affinity iron uptake pathway that was operating under such circumstances. Though little is known about the low affinity uptake system, it is prevalent in microorganisms since deletion of the high affinity system is not lethal and does not impair growth except for some unusual circumstances.

1.1.2.2 High affinity iron uptake system

The high affinity iron uptake system involves the synthesis of specific ligands by microorganisms as scavenging agents and their matching membrane associated receptors (Neilands, 1981a). These compounds are termed as “SIDEROPHORES” (Greek for iron bearers) (Lankford, 1973). Siderophores are secreted into the extra-cellular medium, where they bind Fe$^{3+}$ with high affinity (e.g. $K_a = 10^{-49} \text{M}$ for ferric-enterobactin (Loomis and Raymond, 1991)) and are subsequently transported back into the bacteria as iron siderophore complexes. In contrast to low affinity, the high affinity system has been detected in virtually every aerobic and facultatively anaerobic microbial species (Neilands, 1984).
1.2 SIDEROPHORES AND THEIR ROLE IN IRON TRANSPORT

Siderophores are viewed as an evolutionary response to the appearance of O$_2$ in the atmosphere with the concomitant oxidation of Fe$^{2+}$ to Fe$^{3+}$ and the precipitation of latter as ferric hydroxide. This theory supports the conspicuous absence of strict anaerobes among siderophore producing organisms as the iron requirement under anaerobic conditions could possibly be satisfied with the relatively soluble Fe$^{2+}$ that is present in the growth environment (Neilands, 1981b). Siderophores can be defined as a “low molecular weight virtually ferric specific ligands designated for the solubilization and transport of iron (III) in microbial species”.

1.2.1 Types of siderophores

Unlike enzymes and other macromolecules, which remain almost constant throughout the range of the microorganisms, great variation is seen in siderophore structure from one species to another. More than 500 distinct siderophores have been reported to be secreted by microorganisms and their iron ligation groups have been classified into three main chemical types: hydroxamate, catecholate, hydroxyacid or mixed ligands (Neilands, 1982; Neilands, 1984; Wandersman and Delepelaire, 2004) (Fig. 1.1).

Fig 1.1: Chemical structures of the iron binding groups of the three types of siderophores. (A) Catecholate; (B) Hydroxamate; and (C) Carboxylate.

1.2.1.1 Hydroxamate siderophores

Hydroxamate siderophores contain secondary hydroxamate groups [C(=O)N-(OH)R], where R is an amino acid or derivative of one. Each hydroxamate group provides two oxygen atoms which can complex with Fe$^{3+}$. Hence, if a compound has three hydroxamate groups

Construction of a transgenic *Rhizobium* for *Cajanus cajan* (pigeon pea) (By cloning and expression of a genomic region encoding iron siderophore receptor)
then all six octahedrally deployed coordination sites of the Fe\(^{3+}\) ion can be occupied. Table 1.1 shows various families of microbial hydroxamate siderophores and their sources.

Table 1.1: Some hydroxamate siderophores and their sources. Most of them have been taken from Dexter, 1999 except for the ones whose references are given in bracket.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of Compound</th>
<th>Components</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ferrioxamine</td>
<td>N-hydroxyl-L-ornithine (chain form)</td>
<td>Actinomyces spp., Streptomyces pilosus.</td>
</tr>
<tr>
<td>2</td>
<td>Ferrichrome</td>
<td>Tripeptide of N-acyl-N-hydroxyl-L-ornithine (ring form)</td>
<td>Aspergillus, Penicillium, Neurospora, Ustilago maydis, Ustilago sphaerogena</td>
</tr>
<tr>
<td>3</td>
<td>Schizokinen</td>
<td>Derivatives of citrates. N-hydroxyl-L-acetyl ornithine citrate</td>
<td>Bacillus megaterium. (Byers et al., 1967)</td>
</tr>
<tr>
<td>4</td>
<td>Aerobactin</td>
<td>Derivatives of citrates. N-hydroxyl-L-ornithine citrate</td>
<td>Aerobacter aerogenes (Gibson and Maggrath, 1969)</td>
</tr>
<tr>
<td>5</td>
<td>Rhodotorulic acid</td>
<td>Diketopiperazine of N-δ-acetyl-L-N-δ-hydroxymorphine.</td>
<td>Rhodotorulla pilimanae, Neurospora</td>
</tr>
<tr>
<td>6</td>
<td>Mycobactin</td>
<td>-</td>
<td>Mycobacterium (Barclay and Ratledge, 1983)</td>
</tr>
<tr>
<td>8</td>
<td>Dimerumic acid</td>
<td>-</td>
<td>Fusarium dimerum</td>
</tr>
<tr>
<td>9</td>
<td>Coprogen</td>
<td>-</td>
<td>Neurospora penicillium species</td>
</tr>
<tr>
<td>11</td>
<td>Pyoverdine and pseudobactins</td>
<td>-</td>
<td>Fluorescent pseudomonos (Meyer and Stintzi, 1998)</td>
</tr>
<tr>
<td>12</td>
<td>Gonobactin &amp; nocobactin</td>
<td>-</td>
<td>Neisseria gonorrhoeae, N. meningitidis</td>
</tr>
<tr>
<td>13</td>
<td>Nocobactin</td>
<td>-</td>
<td>Nocardia spp.</td>
</tr>
</tbody>
</table>

1.2.1.2 Catecholate / Phenolate siderophores

These are generally amino acid conjugates of 2, 3-dihydroxy benzoic acid. Each catechol group provides two oxygen atoms for chelation with iron so that a hexadental octahedral complex is formed. The catecholate compounds can be either cyclic or linear. Example of a
cyclic molecule includes enterobactin isolated from *Escherichia coli* (Pollack and Neilands, 1970). Agrobactin isolated from *Agrobacterium tumefaciens* (Ong et al., 1979) and parabactin from *Micrococcus denitrificans* (Peterson and Neilands, 1979) are linear catechols. Table 1.2 lists some catecholate type of siderophores and their sources.

**Table 1.2:** Some catecholate siderophores and their sources

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of Compound</th>
<th>Components</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Enterobactin</td>
<td>DHBA + serine</td>
<td><em>Aerobacter aerogens</em>&lt;br&gt; <em>S. typhimurium</em>&lt;br&gt; <em>E. coli</em> K12</td>
</tr>
<tr>
<td>2</td>
<td>Agrobactin</td>
<td>DHBA + spermidine</td>
<td><em>Agrobacterium tumefaciens</em></td>
</tr>
<tr>
<td>3</td>
<td>Itoic acid</td>
<td>DHBA + glycine</td>
<td><em>Bacillus subtilis</em> (Ito, 1993)</td>
</tr>
<tr>
<td>4</td>
<td>Azotochelin</td>
<td>DHBA + glycine</td>
<td><em>Azotobacter vinelandii</em>&lt;br&gt; (Olga et al, 1984)</td>
</tr>
<tr>
<td>5</td>
<td>2,3 dihydroxy-N-benzoyl-L-serine</td>
<td>DHBA + lysine</td>
<td><em>Azotobacter vinelandii</em>&lt;br&gt; (Olga et al, 1984)</td>
</tr>
</tbody>
</table>

DHBA: Dihydroxybenzoic acid

### 1.2.1.3 Citrate /Mixed Ligand type of siderophores

In siderophores of citrate family schizokinen, arthrobactin, aerobactin and the pseudobactins are grouped. These are called **citrate hydroxamates**. They exhibit citric acid as a common binding block and their α-hydroxycarboxylate group participates in iron binding. Representatives of this family are aerobactin and orthrobactin from *Aerobacter aerogenes* and *Arthrobacter* strains respectively. Schizokinen contains only two hydroxamate groups, the third chelating group being provided by citrate moiety.

However there is no known siderophore in this type in which this bidentate ligand, has relatively modest affinity for iron, and stands alone without support from either hydroxamate or catecholate functional groups.

*Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea)*  
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Mycobactins, agrobactin, parabactin and vibriobactin also have two hydroxamine groups and third pair of chelating group being provided by an oxygen atom on the aromatic residue (o-hydroxyphenyl) and nitrogen on the oxazoline ring.

_Pseudomonas_ sp. and _Azotobacter_ sp. produce a variety of fluorescent chromopeptide siderophores termed pseudobactins and pyroverdine. From studies it appears that these strains produce chemically different pyoverdines, with certain features in common. The pyoverdines have a chromophore which confers color, linked to a small peptide, which differs among strains by the number and composition of amino acids. Figure 1.2 shows the structures of representative examples of each of the three major classes of siderophores.

![Fig 1.2](image_url)

**Fig 1.2** The representative structure of the three major classes of siderophores (A) Ferrichrome, a hydroxamate-type siderophore synthesized by fungi (B) Enterobactin, a catecholate produced by _E. coli_. (C) Pseudobactin B10, a pyoverdin synthesized by _Pseudomonas_ species.

### 1.2.2 Siderophore metal complexes

Within the siderophore series, the affinity (specificity) for Fe$^{3+}$ is a consequence of the pairing of the ‘hard’ acid (relatively small radius/charge) cation with a correspondingly hard base, such as oxygen. The result is an inordinately stable, high-spin d$^3$ exchangeable coordination compound.

Although siderophores have a high affinity ($K_p \approx 10^{30}$) for ferric iron (Crumbliss, 1991), they do form complexes with metals other than Fe$^{3+}$, although with a lower affinity. The
formation constants for the complexes of the hydroxamate siderophore desferriferrioxamine B with Ga$^{3+}$, Al$^{3+}$, and In$^{3+}$ are reported to be in the range of $10^{20}$ to $10^{28}$ (Evers et al., 1989). The stability constants of fluorescent siderophore pseudobactin PSB$_3$ with Zn$^{2+}$, Cu$^{2+}$, and Mn$^{2+}$ range between $10^{17}$ and $10^{22}$ (Chen et al., 1994). B. megaterium ATCC 19213 has been reported to grow well in medium containing 0.1 to 10 mg of aluminum per liter. At low aluminum concentrations, the Al-schizokinen complex has been shown to get transported through the siderophore uptake receptor, resulting in increased cellular aluminum concentrations (Xicheng and Gregory, 1996). Recent laboratory and field studies suggest that Mn$^{2+}$ forms persistent aqueous complexes with high-affinity ligands like desferrioxamine B (Duckworth and Sposito, 2005). Six transposon tagged mutants of P. fluorescens have been reported to show increased gene expression at elevated concentrations of zinc of which, genetic and biochemical analysis revealed that, in four the transposon had inserted into genes essential for the biosynthesis of the siderophore pyoverdine (Rossbach et al., 2000). The presence of metals other than iron has been known to stimulate siderophore formation in a number of bacteria and fungi (Huyer and Page, 1988; Hofte et al., 1993).

1.2.3 Siderophore biosynthesis (Crosa and Walsh, 2002)

The presence of a peptidic backbone in siderophores in combination with the non-proteogenic amino acid unit suggested that these compounds were non-ribosomal peptides. The above statement has been validated by the microbial genome sequencing efforts, and many of the proteins involved in the biosynthesis of siderophores have been found out to be non-ribosomal peptide synthetases (NRPS). Such enzymes were originally identified as catalyzing the syntheses of antibiotics and other substances in gram-positive bacteria.

NRPS are multimodal enzymes that produce peptide products of a particular sequence without an RNA template. Instead, the order of monomeric amino acids activated and incorporated is specified by the order of NRPS domains. The elongating chains grow as a series of acyl-S-enzyme intermediates, tethered covalently to the NRPS assembly line via peptidyl carrier proteins domains (PCPs) that act as way stations for catalytic domains in the vicinity, to carry out chemical operations before the chain is translocated down to the next downstream carrier protein domain. The tethering thiol groups are posttranslationally
introduced as phosphopantetheinyl arms, and the NRPS assembly lines run by a multiple-thiol templating process. Non-ribosomal peptides made by such enzymatic assembly lines include major antibiotics such as the penicillins and vancomycin, the immunosuppressant cyclosporine, and many of the siderophores.

1.2.3.1 Non Ribosomal Peptide Synthetases (NRPS)

An NRPS assembly line is comprised of autonomously folding domains, bundled together into functional modules to carry out steps of monomer selection and activation, chain elongation, and then chain termination. Each NRPS assembly line needs to be organized to carry out four kinds of catalytic operations.

First, each of the peptidyl carrier proteins (PCP) domains must be converted from the apo form to the holo form, bearing the phosphopantetheinyl arm. This is posttranslational priming that must occur on each carrier protein for chain growth to proceed into a full-length product. The enzyme dedicated for this is called phosphopantetheinyl transferase (PPTase) and is encoded in the siderophore biosynthetic gene cluster (Lambalot et al., 1996). PPTases catalyze the attack of a side chain serine in a consensus sequence of the carrier protein domain on the pyrophosphate linkage of coenzyme A, transferring the phosphopantathenate (P-pant) moiety, yielding the phosphodiester linkage to the PCP serine side chain and releasing 3',5'-ADP as soluble product (Fig. 1.3A).

Second, the assembly line must be able to select and activate monomers and incorporate them at particular positions in the growing chain. The carboxylic acids and amino acids to be activated are selected by adenylation (A) domains, operating with logic analogous to that of aminoacyl tRNA synthetases: the amino acid selected by the A domain active site is converted via attack on the co-substrate ATP to the aminoacyl-AMP (Fig. 1.3B). This thermodynamically activated monomer is then transferred to the adjacent HS-pant-PCP domain, to covalently attach to the aminoacyl moiety on the thiol and form aminoacyl thioesters (aminoacyl-S-PCP).
The third step of the biopolymer assembly line is chain elongation and is shown in Fig. 1.3C (von Dohren et al., 1997; Cane et al., 1998; Welch, et al., 2000). In the NRPS superfamily of enzymes, siderophore chain growth is directional, from the most upstream, N-terminal PCP domains, to the most downstream, C-terminal PCP domains. Every peptide bond-forming step is tied to chain elongation and translocation. The peptide synthetase catalytic domains are termed condensation (C) domains. The actual peptide bond-forming step by each C domain involves an upstream peptidyl-S-PCP as the donor co-substrate and the proximal downstream aminoacyl-S-PCP as the attacking, acceptor substrate. In this way the translocated chain grows by one peptide bond and is ready for another elongation cycle by the C domain of the next downstream module (Fig. 1.3C).
Fig 1.3: Initiation of siderophore synthesis. (A) The priming activity of PPTase, (B) The two-step adenylation domain equation, (C) Equations of condensation domain.
The fourth and the last step are the chain termination (Fig 1.4) and release of the full-length non-ribosomal peptide or siderophore (Keating et al., 2001). Because the peptidyl chains grow as a cascade of elongating acyl-S-enzyme intermediates, when the full-length chain arrives at the most-downstream PCP domain, it is still covalently docked and requires chemical cleavage from the enzyme assembly line. In majority of NRPS the carboxyl terminal domain is a 30 to 35-kDa autonomously folding unit termed as thioesterase (TE), Chain release involves interdomain transfer from the PCP to the active site serine side chain in the active site of the TE to yield an acyl-O-TE intermediate (Rusnak, et al., 1991). This intermediate can experience two major fates: intermolecular hydrolysis, to release the free acid (e.g., the TE of ACV synthetase), or intramolecular cyclization causing capture by an OH or NH$_2$ group in the peptidyl chain, to release a cyclic lactone or lactam (e.g., enterobactin or gramicidin).

A substantial variety of siderophore structures are thus produced from similar NRPS assembly lines. Variation can come in the choice of phenolic acid selected as N-cap, the
tailoring of amino acid residues during chain elongation, and the mode of chain termination and the nature of the capturing nucleophile of the siderophore acyl chain being released. The specific parts that get assembled and tailored in a given bacterium may reflect a combination of siderophore biosynthetic gene cluster that are available and studied till now.

1.2.4 Ferri-siderophore uptake machinery
Siderophores have been designed by nature in such a way that they are of sufficient size to engage the six octahedrally directed valence bonds of Fe (III), thus they exceed the free diffusion limit of the small water-filled pores in the outer membrane of enteric bacteria. This in turn was sufficed by nature by the evolution of specific receptors for the recognition and transport of the iron-laden form of the siderophore. A siderophore system is thus comprised of two principal parts; the ligand, and an arrangement for its uptake and utilization. Both parts of system are mostly subject to a common regulatory device triggered by the intracellular concentration of iron. As shown in Fig 1.5 the ferric-siderophore (Sid-Fe$^{3+}$) from the extracellular medium is recognized by the N-terminal portion of a ferri-siderophore receptor, which serves two functions. First, the ferri-siderophore receptor transports Sid-Fe$^{3+}$ into the periplasm, which is further transported into the cytoplasm by an ABC transporter. Both transport and induction functions require energy transduction from the TonB–ExbB–ExbD complex in the inner membrane (purple). Sid-Fe$^{3+}$-bound ferri-siderophore receptor is believed to interact with TonB via its TonB-box motif.
1.2.4.1 Iron regulated outer membrane proteins (IROMPs)

Much has been learned about the mechanism of iron acquisition and transport in Gram negative bacteria, especially *E. coli*. It has been shown that transport across the outer membrane of Gram negative bacteria requires receptor proteins with a high specificity for a particular Fe$^{3+}$ siderophore. Seven such Fe$^{3+}$ siderophore receptor proteins have been identified in the outer membrane of *E. coli*, each recognizing a certain Fe$^{3+}$ siderophore or a group of structurally very similar siderophores (Braun, 1995).

Outer membrane receptors are essential for the uptake of ferric siderophore complexes, vitamin B12 and colicins. These substrates are usually too large for the channels of type I
and II porins, present in very low concentration and necessitating the use of specific cell surface receptors to govern uptake. These receptors binds the ligands with a much higher affinity (Km = 0.2μM) than, for example, LamB of the type II porins (Km = 0.1 mM). In *E.coli*, the ferric complexes of ferrichrome, coprogen, aerobactin, enterochelin, dihydroxybenzoic acid (DHBA) and citrate, bind specifically to the outer membrane proteins, without any receptor cross-reactivity, to FhuA, FhuE, Iut, FepA, Fiu, Cir, Irr, FecA respectively. These OMPs are expressed under iron limiting conditions and hence are called iron regulated outer membrane protein (IROMPs). Their molecular weight varies between 75-85 kDa. IROMPs from various organisms are shown in Table 1.3.
Table 1.3: IROMPs from various organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Siderophore class</th>
<th>IROMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Hydroxamate</td>
<td>FhuA</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Hydroxamate</td>
<td>PhuE</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Hydroxamate</td>
<td>UntA</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Catecholate</td>
<td>FepA</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Catecholate</td>
<td>Fiu</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Catecholate</td>
<td>Cit</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Catecholate</td>
<td>FecA</td>
</tr>
<tr>
<td><em>B. japonicum</em></td>
<td>Catecholate</td>
<td>FecA</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>Pyoverdine</td>
<td>FpvA</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>Pyochelin</td>
<td>FptA</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>Pseudobactin</td>
<td>PupA</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>Pseudobactin</td>
<td>PbuA</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>Hydroxamate</td>
<td>PrgA</td>
</tr>
</tbody>
</table>

Three of the outer membrane receptors studied so far like FhuA, FepA and FecA, using X-ray crystallography consists of anti-parallel β-strands that form a β-barrel. They contain a globular domain that inserts into the β-barrel from the periplasmic side and tightly closes the channel formed by the β-barrel. More than half of the molecule is located above the outer-membrane lipid bilayer. The Ton B box linked to the globular domain has been documented for five outer membrane transporter proteins studied including FecA. Ton B induced conformational change in the β-barrel and in the globular domain release the substrates bound and open the channel (Braun and Braun, 2002).
1.2.4.2 Proposed mechanisms of ferri-siderophore uptake by IROMPs

Till now two mechanisms have been proposed for the of ferrisiderophore complex uptake. They are direct uptake of siderophore using ferrisiderophore receptor and the shuttle mechanism.

1.2.4.2.1 Role of TonB protein in outer membrane mediated transport

Transport of all Fe$^{3+}$ siderophores across the outer membrane requires energy (Koster and Braun, 1990). The TonB system of gram negative bacteria appears to exist for the purpose of transducing the energy generated by the proton motive force at the CM, to the OM, where it is needed for active transport of iron siderophores, vitamin B12 and in pathogens iron from host binding proteins (Reigh and O'Connell, 1993; Moeck and Coulton, 1998; Postle and Kadner, 2003). In *E. coli* it was found by the analysis of *tonB* mutants, that the TonB protein is essential for ferri-siderophore transport (Frost and Rosenberg, 1975; Hantke and Braun, 1978). TonB is a 36kDa protein located in the cytoplasmic membrane (Postle and Good, 1983). This protein is also required for the transport of vitamin B12, group B colicin, as well as infection by bacteriophage T1 and Φ80 (Braun et al., 1998).

The TonB dependent receptors achieve maximal transport efficiency at very low concentrations of ligand (Neilands, 1982). This is achieved by the interaction of TonB with components in both the CM and the OM, and its accessory proteins ExbB and ExbD, which are anchored in the CM. The available data suggests that ExbB and ExbD use the proton motive force (pmf) of the CM to convert TonB to an energized conformation that is subsequently transduced by TonB to the OM transporters (Ferguson et al., 2002; Held and Postle, 2002; Ogierman and Braun, 2003; Postle and Kadner, 2003). TonB and ExbD possess single transmembrane (TM) segments near their N-terminal ends, with the bulk of their sequences in the periplasm. ExbB has three TM segments, oriented with the bulk of the protein in the cytoplasm. All the three proteins act as a complex. The ExbB appears to stabilize both TonB and ExbD (Postle and Kadner, 2003).

*Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea)*

*By cloning and expression of a genomic region encoding iron siderophore receptor*
The TonB protein can be divided into several functional domains and contains several conserved sequence motifs. The first is the N-terminal TM region, which has a direct role in the energy transduction process. Two residues in the TM domains, Ser-16 and His-20, are essential for both the ExbB/D-mediated conversion of TonB to its energized form and OM active transport. The interaction of TonB amino acid 160 with the Ton box of the OM transporter is required for ligand transport. Just after this is the region of C-terminal domain, is the region which is required for the OM association (Postle and Kadner, 2003).

1.2.4.2.2 The siderophore iron shuttle mechanism:
An alternative mechanism is proposed by Stintzi et al. (2000) to explain transport in Aeromonas hydrophila is found to occur by means of an indiscriminant siderophore transport system composed of a single multifunctional receptor. In this mechanism the iron free siderophore was considered to be in large excess over the iron-loaded siderophore. The iron-loaded siderophore is brought close enough to iron free bound siderophore to promote iron exchange. The ligand exchange induces a conformational change, which indicates the iron-loaded status of bound siderophore to the TonB protein. Energized TonB triggers a conformational change of the receptor allowing translocation of the ferric siderophore from cell surface to periplasmic space. The ferric siderophore that gave up its iron then binds to the receptor replacing the transported ferric siderophore complex. Finally, the receptor returns to its initial conformation. The net result is a shuttle mechanism in which siderophore ligands pass serially through a channel with iron exchange between siderophores a key step in the process. This process is illustrated in Fig. 1.6.
1.2.4.3. Periplasmic binding proteins

Periplasmic binding proteins (PBPs) recognize and bind the iron-siderophore complex that has been actively transported by the outer membrane receptor proteins into the periplasm. PBPs are a necessary component for transport of the iron-siderophore complex into the cytoplasm. These proteins are generally synthesized at a lower level compared to PBPs that bind amino acids or sugars and have a lower affinity for ferri-siderophore complexes as compared with OM receptors (Sprenzel et al., 2000). They undergo a conformational change upon binding of the substrate. Periplasmic binding proteins are not specific for single metal chelates and can bind to more than one type of siderophore complex. An example of a periplasmic binding protein is FhuD, which shuttles all hydroxamate siderophores that have been imported by the outer membrane receptor proteins FhuA, FhuE, and IutA to a single ABC-type transporter. The crystal structures of both PBPs FhuD (ferrichrome) and BtuF (vitamin B12) are known (Clarke, 2000; Borths et al., 2002; Karpowich et al., 2003) and FepB is the periplasmic binding protein for ferric enterobactin (Chakraborty et al., 2003).

The PBP-ferric siderophore complex provides a substrate for the next component of these iron transport systems, the ATP-dependent ABC-type transporter. PBPs are not involved in the transport of ferrisiderophore complexes in all the organisms for example in E. coli.
ferrichrome transport is an Fhu-PBT (Fhu-Periplasmic Binding protein dependent Transport) system but in *B. japonicum*, transport of ferrichrome is through a two-component system (Benson et al., 2005). FegA transports it from outside to the periplasm and a specialized permease FegB transports it from periplasm to the cytoplasm. No periplasmic binding protein is involved to carry the ferrisiderophore complex to the inner membrane protein. A similar system of transport of rhizobactin occurs in *S. meliloti* (O'Cuiv et al., 2004). This transport system is referred to as “Periplasmic binding protein independent”.

1.2.4.4. Transport across the cytoplasmic membrane

In contrast to transport across the outer membrane, transport of Fe$^{3+}$ siderophores, heme and iron across the cytoplasmic membrane is driven by ATP hydrolysis. The most common substrate transport mechanism in bacteria – through ABC transporters – is used for all iron sources (Braun et al., 1998). Iron, Fe$^{3+}$ siderophores and heme bind to proteins that deliver the compounds to integral cytoplasmic membrane proteins. Deletion of the genes that encode these binding proteins inactivates transport. An ABC transporter that is encoded by the *sfuABC* genes and transports apparently unchelated Fe$^{3+}$ across the cytoplasmic membrane was first characterized in *Serratia marcescens*. Proteins encoded by homologues of *sfuABC* were then shown to transport iron into *N. gonorrhoeae* (the *fbpABC* genes), *H. influenzae* (the *hitABC* genes), *Y. enterocolitica* (the *yfuABC* genes), and *Actinobacillus pleuropneumoniae* (the *afuABC* genes) (Braun et al., 1998).

*In vitro* studies of reconstituted maltose- and histidine-transport systems have demonstrated that substrate-loaded binding proteins trigger ATP hydrolysis (Boos and Lucht, 1996). Such a mechanism prevents wasteful ATP hydrolysis when no transport substrates are present. Triggering of ATP hydrolysis implies that substrate-loaded binding proteins contact the ATPases directly or that a signal is transmitted through the integral transport protein from the periplasm to the cytoplasm. Given that ATP is provided in the cytoplasm, the ATP-binding sites of the ATPases must be exposed to the cytoplasm.

FhuC is the ATPase used by the ferrichrome transport system. Few distinct regions of FhuB interact with FhuD (shown in red and as red asterisks in Fig.1.7) (Mademidis et al., 1997).
These regions correspond to segments exposed to the periplasm and, unexpectedly, to transmembrane segments and the cytoplasmic adjoining regions (Mademidis et al., 1997). Region 7 is probably a binding site for the FhuC ATPase (Köster, 1991). The figure emphasizes the transmembrane topology of FhuB. The loops probably fold back into the FhuB structure; they might be part of an FhuB channel into which FhuD inserts from the outside, and FhuC inserts from the inside. In such a model, FhuD comes into close contact with FhuC and might activate FhuC through a direct interaction. (Bruns et al., 1998).

Fig 1.7: The arrangement of the FhuABCD transport proteins that transport ferrichrome into the cytoplasm of *Escherichia coli* K-12. FhuA forms a β-barrel that has a closed channel that is opened by interaction with the Ton system. Ferrichrome binds to the FhuD protein, which delivers ferrichrome to the FhuB protein in the cytoplasmic membrane. The sites of interaction between FhuD and FhuB are shown in red and by red asterisks. The FhuC ATPase drives transport through FhuB.

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*By cloning and expression of a genomic region encoding iron siderophore receptor*
1.2.5 Genetic organization of siderophore biosynthesis and uptake genes

Most of the siderophore biosynthetic and uptake genes are clustered and organized into an operon. For example, the \textit{rhhABCDEF} comprise an operon that functions in the regulation, biosynthesis, and transport of rhizobactin1021, a hydroxamate siderophore produced under iron stress by \textit{Sinorhizobium meliloti} 2011 (Lynch et al., 2001). In \textit{E. coli} genome the iron uptake systems are encoded by more than 20 genes that form several operons, the aerobactin biosynthesis and uptake system \textit{iucABCDiutA} (Braun and Hantke, 1991); the ferrichrome uptake system \textit{fhuACDB} (Fecker and Braun, 1983); the uptake/sensory system for the ferri-dicitrate complexes by the \textit{fecIRABCDE} operon (van Hove B et al., 1990) and large locus containing genes for enterobactin biosynthesis (\textit{entABCDEFG}) and uptake (\textit{fepABCDEG}) organized in five small operons (Panina, et al., 2001) (Fig 1.8). Similarly biosynthesis of pyochelin requires the iron-regulated \textit{pchDBCA} operon in \textit{P. aeruginosa} (Serino et al., 1997).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig1.8}
\caption{Genetic organization of enterobactin biosynthesis and transport genes in \textit{E. coli}.}
\end{figure}

1.2.6 Regulation of ferri-siderophore iron assimilation system

The iron content of cells must be regulated to conserve energy and substrates, and to avoid iron toxicity. The easy one electron conversion of Fe$^{2+}$ to Fe$^{3+}$ results in the formation of oxygen radicals (Arroyo et al., 1994; Touati, 2000). of which the hydroxyl radical is the most active radical in the oxidative destruction of DNA, lipids and proteins.. A simple method to reduce radical formation by iron is to limit the availability of the iron atom itself; by sensing adequate iron levels and limit its uptake. Synthesis of iron-transport proteins and siderophore biosynthesis enzymes is indeed repressed by iron. Under iron depleted growth conditions, synthesis can increase 30-fold.

\begin{center}
\textit{Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea) By cloning and expression of a genomic region encoding iron siderophore receptor}
\end{center}
In the majority of Gram-negative organisms, 'Fur' (ferric uptake regulator) protein is the key regulator for expression of genes involved in iron transport (Hantke 1981). Fur is a transcriptional repressor of more than 90 different genes in for eg, *E. coli* and *Pseudomonas aeruginosa*, many of which are involved in siderophore synthesis and uptake (Wexler et al., 2003). In environments where iron is abundant, the Fur protein (in its repressive mode) attached to Fe$^{2+}$ binds fur boxes, blocking transcription of target genes (Masse and Gottesman, 2002). In *E. coli*, all known siderophore systems are negatively regulated by Fur (Hantke K. 1981). At low concentrations of Fe$^{2+}$, the Fur protein has a weak affinity for operator DNA. At high Fe$^{2+}$ concentrations, Fur binds tightly to operator DNA (De Lorenzo et al., 1987). A simple schematic representation of this has been shown in Fig 1.9. In addition to Fe, several other divalent cations, including Mn, Co, and Cu, bind to Fur (De Lorenzo et al., 1987). Indeed, resistance to manganese has formed the basis for selection to obtain Fur mutants of several gram-negative bacteria (Hantke, 1987; Prince et al., 1993; Tolmasky et al., 1994). High concentrations of manganese presumably stop cell growth by repressing iron supply systems. The site at which Fur binds to DNA has been termed an iron box or a Fur box, binding of Fur to the iron box has been shown experimentally for several genes, including *iucA* (De Lorenzo et al., 1987; De Lorenzo et al., 1988a), *cir* (Griggs and Konisky, 1989), and *fur* itself (De Lorenzo et al., 1988b).

All of the deduced Fur proteins show a high degree amino acid similarity. Indeed, *fur* from *P. aeruginosa* can functionally complement an *E. coli* fur mutant (Prince et al., 1993) as can *fur* from *N. gonorrhoeae* (Berish et al., 1993), *Y. pestis* (Staggs and Perry 1991), *V. vulnificus* (Litwin and Calderwood, 1993), and *V. cholerae* (Litwin et al., 1992). Although originally identified as a repressor of iron transport and siderophore biosynthesis, fur has also been reported to regulate genes involved in other functions, including oxidative stress, energy metabolism, and virulence, suggesting that defects in Fur regulation could have serious consequences for a microorganism (Hantke, 2001).

In some gram-positive bacteria, Fe-responsive gene regulation is mediated by members of the DtxR family, identified in *Corynebacterium diphtheriae* as a regulator of Fe-dependent diphtheria toxin (Boyd et al. 1990). This protein also uses ferrous iron as a corepressor (Qian...
et al., 2002) but shows no sequence homology to Fur (Wexler et al., 2003). DtxR protein in Gram positive bacteria represses gene transcription when loaded with Fe\(^{2+}\) (Braun et al., 1998). Fur and DtxR share very little sequence similarity; however, the N-terminal DNA-binding sites and the metal-binding domains might assume similar structures. Both proteins are active as dimers. Fur and DtxR regulate transcription of bacterial toxin genes, including those that encode *Serratia marcescens* hemolysin, *Shigella dysenteriae* shiga toxin, *Pseudomonas aeruginosa* exotoxinA and diphtheria toxin. Fur and DtxR are the principal iron-regulatory proteins and mainly act negatively, repressing gene transcription when cells iron requirements are met. In addition to Fur, there are at least three other subgroups in the Fur superfamily. PerR is an oxidative-stress-response regulator (Bsat et al., 1998) and Zur regulates genes involved in Zn uptake (Hantke, 2001). The biology of these repressor proteins has been reviewed (Escolar et al., 1999; Hantke, 2001).

In *E. coli*, siderophore systems are coordinately derepressed under iron deficiency, regardless of whether any particular siderophore is present. In many species expression of iron uptake genes requires transcriptional activation (Crosa, 1997). This review focuses on a regulatory mechanism involving extracytoplasmic function (ECF) sigma factors that are required for expression of siderophore synthesis and uptake genes. The involvement of alternative sigma factors provides an additional level of regulation of siderophore uptake, enabling bacteria to respond to the presence of specific siderophores in the environment as well as to levels of intracellular iron. The ferric dicitrate system exemplifies such a system, in which induction of *fecABCDE* gene transcription requires exogenous ferric dicitrate (Zimmermann et al., 1984). Under iron-limiting growth conditions and in the presence of ferric citrate, synthesis of a ferric-citrate transport system is induced. The ferric citrate-transport system is a typical Gram-negative Fe\(^{3+}\)-siderophore-transport system in which FecA plays a dual role: it transports ferric citrate across the outer membrane into the periplasm, and it is required for initiation of *fecABCDE* gene transcription. The *fecR* gene encodes a protein that spans the cytoplasmic membrane once and, thus, could transmit information across it. Cells that synthesize only the N-terminal portion of FecR, which is located in the cytoplasm, constitutively transcribe the *fec* transport genes. This N-terminal fragment might assume the conformation that it adopts in the complete FecR protein upon
ferric-citrate induction. FecR does not interact with DNA; rather, it activates FecI, the second Fec regulatory protein. FecI is a sigma factor and, when activated, binds to a promoter upstream of the *fecA* gene (Fig. 1.7). FecI seems to regulate only *fec* transport-gene transcription. The level of intracellular iron regulates, through Fur, transcription of the *fecR* and *fecl* genes and, in addition, regulates transcription of the *fec* transport genes directly. This is an considered as an economic method of gene regulation because the regulatory proteins are synthesized only when iron is needed. When sufficient levels of iron are present in the cells, transcription of transport genes is repressed immediately by Fur; cells do not wait until the inducing proteins are diluted out during growth.

In *P. putida*, iron deficiency induces the synthesis of pseudobactin 358 and of PupA, its outer membrane receptor protein; however, the regulatory gene, *pfrA*, controls the synthesis of the biosynthetic genes but not that of *pupA* (Venturi et al., 1993). PvdS and FecI are part of a distinct subfamily of ECF sigma factors involved in iron acquisition and hence named the iron-starvation sigmas. Analysis of microbial genome sequences showed that Fec-like signalling systems are present in a wide range of species and many such systems may be present in a single species (Visca et al., 2002).

Transport of the ferric-ligand is apparently not necessary for transcription induction. Recent biophysical and crystallographic experiments have shown that a subclass of TonB-dependent iron transporters, which can be considered as ‘metal detectors’ (Buchanan, 2005) can bind iron-free ligands and it is speculated that the binding of iron-free siderophore initiates the signal transduction cascade (Schalk et al., 2004).

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Chapter 1: Review of Literature

HIGH IRON
Repression of iron uptake genes

LOW IRON
Derepression of iron uptake genes

apo-Fur

Fig. 1.9 Schematic representation of Fur-mediated gene repression (Andrews et al, 2003).

1.3 FERRIC HYDROXAMATE UPTAKE (Fhu) SYSTEM

The ferric-hydroxamate siderophore complexes in E. coli are transported using an OMP called FhuA. FhuA is a 78kD multifunctional protein in the outer membrane of Escherichia coli. It’s an outer membrane receptor for ferric hydroxamate uptake, an integral membrane protein composed of 714 amino acid residues (Coulton et al., 1986). It transports Fe$^{3+}$ chelated to hydroxamate siderophores such as ferrichrome and its structural analog ferricrocin. In addition to binding of the ferric siderophore ferrichrome-iron, FhuA also serves as the primary receptor for several bacteriophages (T1, T5, Φ80 and UC-1), the bacteriotoxin colicin M and the antibiotics albomycin, microcin J25 and CGP 4832 (Ferguson et al., 2000; Braun et al., 2002). The binding of ferrichrome-iron to FhuA induces conformational changes (Moeck et al., 1996) signalling the ligand-loaded status of the receptor. This signal seems to be a requirement for TonB-dependent energy transduction (Moeck et al., 1997). The crystal structures of unliganded FhuA and of FhuA in complex with ferrichrome (Ferguson et al., 1998; Locher et al., 1998) allow a deep insight into the molecular architecture of TonB-dependent receptors as well as into their mechanism of ligand binding.

1.3.1. Structure of FhuA (Ferrichrome Receptor):

The crystal structure of the FhuA protein has been determined by Ferguson et al. in 1998 (Ferguson et al., 1998). This was the first crystal structure of a protein–lipopolysaccharide

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25
complex (Ferguson et al., 2000). The hydrogen–bonds and electrostatic interactions between FhuA and the LPS are provided by eight positively charged residues of FhuA. The C-terminal domain, including residues 161–714, forms a 22-stranded antiparallel β-barrel that spans the outer membrane. The β-barrel is closed from the periplasmic side by a domain designated the 'cork' or 'plug' that comprises residues 1–159, which form a four-stranded β-sheet and four short α-helices. The cork completely closes the channel formed by the β-barrel (Figure 1.10). Ferrichrome binds in a pocket slightly above the external outer membrane surface. Four amino acid residues of the cork domain and six residues of the barrel domain come within 4.5Å of ferrichrome so that they can form hydrogen bonds and Van der Waals contacts (Locher et al., 1998; Braun et al., 1999).

![Fig 1.10](image)

Fig 1.10 The overall structure of the FhuA – ferrichrome – LPS complex (Ferguson et al., 1998b). View onto FhuA from (A) side–view (B) the external environment. The ferrichrome receptor FhuA in its ligand-free (C) and ligand-loaded (D) conformations.

1.3.2 The periplasmic protein FhuD can also bind other ferric hydroxamate complexes such as coprogen, ferrioxamine and rhodotorulic acid (Koster and Braun, 1990; Rohrbach, 1995a). The binding pockets of both, FhuA and FhuD contain a concentration of hydrophobic residues that interact with the ornithyl moieties of the siderophores. (Ferguson et al., 1998)
1.3.3 Inner Membrane Associated Proteins
Transport across the inner membrane requires two proteins, one to span the membrane to act as a permease and a second one which can hydrolyze ATP to provide the energy for transport. These proteins must first recognize the cognate ligand-loaded periplasmic protein, extract and transport the ligand across the cytoplasmic membrane while utilizing the energy of the third ATP hydrolyzing protein component.

1.3.3.1 Transmembrane Permease: FhuB
The permease component is typically a hydrophobic protein (65%) (Persson and Argos 1994). The domains bind non-covalently to form a complex (Koster and Braun, 1990). Interaction between the periplasmic protein FhuD and FhuB indicates that the ligand-loaded FhuD preferentially associates with FhuB (Rohrbach, 1995b).

1.3.3.2 ATP-binding proteins: FhuC
FhuC is known to have ATP-hydrolase activity. Interaction between FhuC and FhuB is important for energizing the transport across the inner membrane.

Once inside the cytoplasm, siderophore reductases reduce the ferric iron to Fe^{2+} lowering its affinity for the siderophore (Fishcer et al., 1990). The siderophore then can be recycled to the outside of the cell (Matzanke et al., 1991). In some cases hydrolysis of the siderophore (i.e. enterobactin) has also been reported, which can aid in the release of bound Fe^{3+} (Brickman and McIntosh, 1992).

1.4 FERRIC CITRATE UPTAKE (FecABCDE) SYSTEM:
Ferric-citrate transport in *E. coli* required products of the fecABCDE genes and functional tonB and exbB genes (Pressler et al., 1988; Standenmaier et al., 1989). These genes are negatively regulated by iron, as are the other *E. coli* genes involved in siderophore production and transport (Neilands, 1989). FecA is an OMP with an approximate molecular weight of 80.5kDa (Wagegg and Braun, 1981). The citrate mediated iron uptake in *E. coli* appears to be induced by the presence of citrate in the growth medium (Frost and Rosenberg, 1973). But in case of *Bradyrhizobium japonicum* (Meyer and Abdallah 1978),

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Pseudomonas aeruginosa (Cox, 1980), and Mycobacterium smegmatis (Messenger and Ratledge, 1982) presence of citrate in the growth medium is not essential for the induction of the system. Furthermore in Mycobacterium smegmatis and Rhodopseudomonas sphaeroides the mechanism of iron uptake via ferric-citrate appears to be constitutive (Messenger and Ratledge, 1982; Moody and Dailey, 1984).

1.5 SIDEROPHORE-MEDIATED MICROBIAL INTERACTIONS IN SOIL

1.5.1 Siderophores in soil

Hydroxamate type siderophores are reported to be present in a wide variety of soils in significantly high concentrations. The occurrence of hydroxamate siderophores in soils has been demonstrated in bioassays with Arthrobacter terregens (Lochhead and Burton, 1956) and Arthrobacter flavescens (Powell et al., 1980). Holstorm et al (2004) have identified the siderophores ferrichrome and ferricrocin in soil solution of the upper organic layer by High Performance Liquid Chromatography (HPLC) Mass Spectrometry (MS). Cline and coworkers (Cline et al., 1983) demonstrated that hydroxamate siderophores are able to form stable Fe chelates over the normal pH range of soils, in contrast to a number of other natural chelating agents.

Hydroxamate siderophores of the ferrichrome type are of special ecological interest because their amounts in soils have been quantified and found to be in nanomolar concentrations as estimated by physicochemical method (Holmstrom et al., 2004). By an E.coli based bioassay method, Powell et al (1983) showed that the ferichrome- type hydroxamate siderophores in a 1:1 (soil-water) extract was approximately 78nM by using enterobactin defective E. coli K-12. Nelson et al (1988) used a panel of E. coli auxotrophic mutants to determine enterobactin, ferrichromes, coprogen, and unidentified siderophore in the rhizospheric soil.

Ferrichrome type siderophores are produced by many soil fungi (Zahner et al., 1963), including symbiotic ectomycorrhizal fungi (Szaniszlo et al., 1981), They have the ability to mobilize iron in neutral and alkaline soils in which other naturally occurring compounds are ineffective as iron chelators because of competition from other metal ions (Cline et al., 1982). Experimental evidence indicates that hydroxamate siderophores can supply iron to some plant species (Orlando and Neilands, 1982; Powell et al., 1982). Monoclonal
antibodies (Mabs) have greater potential to detect specific siderophores at lower concentrations therefore this technique has been used for the detection of ferric pseudobactins in rhizospheric samples (Buyer and Sikora, 1990).

1.5.2 Advantages of siderophore production in natural environments
Under natural conditions, the ability to produce siderophores has been demonstrated to confer a selective advantage to the producer organism. Rootlet elongation and bacterial growth on rootlets was determined by De Bellis and Ercolani (2001). After inoculating cucumber and spinach seedlings with *Pseudomonas* strains differing in production of siderophores and HCN, it was shown that the siderophore producers grew more profusely than nonproducers on both species and promoted rootlet elongation on cucumber. Coinoculation of siderophore producers and nonproducers resulted in restricted growth of the latter (De Bellis and Ercolani, 2001). Mirleau et al (2000) have shown that a *P. fluorescens* strain mutant for pyoverdine siderophore (pvd') was significantly more susceptible to iron starvation than the wild type strain (C7R12), also the survival kinetics of mutant and wild type when co-inoculated, showed a bacterial competition more favorable to the pyoverdine producer C7R12. The pvd' *P. fluorescens* mutant was clearly affected in its ability to compete not only in the rhizosphere but also in the bulk soil (Mirleau et al., 2000).

1.5.3 Utilization of heterologous siderophores
Microorganisms which themselves do not synthesize a particular type of siderophore may yet be proficient at the uptake of its iron-bound siderophore complex and may thus utilize iron bound to such ‘heterologous’ siderophores in addition to the siderophores they themselves synthesize i.e. ‘homologous’ siderophores. Utilization of heterologous siderophores is widespread in pathogenic, commensal and saprophytic microorganisms. For e.g., *E. coli* has six known siderophore receptors (Cir, FecA, FepA, FhuA, FhuE, Fiu) providing specificity for several ferri-siderophores (and ferric dicitrate) of which only enterobactin and its derivatives are synthesized endogenously (Hantke, 2001). Fluorescent pseudomonads are known to efficiently colonize various ecological niches, which is largely attributed to the diverse and sophisticated iron uptake systems they possess. They have an ability to utilize a large number of heterologous siderophores via different TonB dependent
siderophore receptors. For instance, in *Pseudomonas aeruginosa* PAO1, in addition to two siderophores, pyoverdine and pyochelin synthesized by this strain can also utilize the heterologous bacterial siderophores coprogen, cepabactin, salicylic acid, desferriferrioxamine B and a number of siderophores produced by other pseudomonads (Meyer, 1992). Genome analysis has shown 32 putative siderophore receptors in *P. aeruginosa* (Dean and Poole 1993; Ankenbauer and Quan, 1994), 29 in *P. putida*, 27 in *P. fluorescens* and 23 in *P. syringae* (Cornelis and Matthijs, 2002). In these cases the ability to cross-utilize heterologous siderophores is accounted for by the presence of multiple type of siderophore receptors (Cornelis and Matthijs, 2002) while in other organisms use of a low specificity system that recognizes more than one type of siderophores may be responsible (Crowley et al., 1991).

Addition of heterologous siderophores has been shown to cause growth stimulation in an alpha proteobacterium strain V0210 (Guan et al., 2000). In natural environments such as the rhizosphere, the capacity to utilize heterologous siderophores produced by other members of the rhizosphere microflora is a positive fitness factor (Raaijmakers et al., 1995; Loper and Henkels, 1999). Utilization of heterologous siderophores is a sound strategy for iron acquisition because siderophores are excreted into the soil where they are freely available and this ability may confer a selective advantage in the rhizosphere.

1.5.4 Competition for iron in rhizosphere

In natural environments there are always competitive interactions among the microbial inhabitants for the available nutrients and the success of an organism depends upon how efficiently it can acquire limiting essential nutrients. Iron being limiting in soil and rhizosphere has been demonstrated by reporter studies which aimed at determining the concentration of ferric iron available to soilborne microflora. Loper and Lindow (1994) have produced a whole-cell iron biosensor consisting of cells of various *Pseudomonas* species harboring a transcriptional fusion of an iron-regulated promoter from a locus encoding the membrane receptor for a pyoverdine siderophore to a promoterless ice nucleation reporter gene. The ice nucleation activity of iron biosensor strains inoculated onto the bean rhizosphere or phyllosphere revealed that, on average, cells sensed Fe$^{3+}$ concentrations that...
were intermediate between iron-replete and iron-deplete culture media (Loper and Lindow, 1994; Marschner and Crowley, 1997). Availability of iron to *P. fluorescens* Pf-5, assessed by the in situ transcription of an iron-regulated promoter, was low immediately following inoculation of the bacterium onto bean roots but typically increased thereafter, since several sources of iron may become available to Pf-5 in the rhizosphere (Loper and Henkels, 1997).

To gain an understanding of the iron distribution on a small scale on plants Joyner and Lindow (2000) developed a whole-cell biosensor to detect and measure bioavailable iron at the level of single bacterial cells. Ferric iron availability to cells of *Pseudomonas syringae* was assessed by quantifying the fluorescence intensity of single cells harbouring a plasmid-borne transcriptional fusion of an iron-regulated promoter from a locus encoding a membrane receptor for a pyoverdine. Their results indicated that there is substantial heterogeneity of iron bioavailability to cells of *P. syringae* on plants, with only a small but significant subset of cells experiencing low iron availability. It is believed that chemical reactions and microbial respiration (Hojberg and Sorensen, 1993) can result in fluctuations in pH which in turn could affect the solubility of iron oxides, ultimately affecting the bioavailability of this element locally (Ehrlich, 1990; Buyer and Sikora, 1991; Loper and Ishimaru, 1991; Marschner, 1995; Loper and Henkels, 1999). These results support models of competition for limiting iron resources among microbes on plants in that at least some of the cells in population encounter very low iron availability, thus competition for this nutrient is an important aspect of rhizosphere ecology.

Competition for iron can be regarded as occurring in two stages; either competition between the excreted siderophores for the metal or competition between microorganisms for the Fe-siderophore complexes that are available in soil. The former is controlled by proton dissociation and formation constants of each siderophore, kinetics of exchange as well as their concentration, while the latter is governed by the existence of an uptake mechanism for, and its affinity to, the Fe complex (Jurkevitch et al., 1992). Fluorescent pseudomonads can suppress various soil borne plant diseases (Weller, 1988). A clear relationship has been established between the suppression of the soil borne fungal diseases by them and their densities in the rhizosphere (Bull et al., 1991; Raaijmakers et al., 1995). Siderophores produced by several of the fluorescent *Pseudomonas* spp. play a role in biological control of...
plant pathogens and in plant growth promotion through competition for Fe (Klopper et al., 1980; Schippers et al., 1987). The major siderophores of the fluorescent pseudomonads are pyoverdines; they have very high affinity for Fe$^{3+}$ with a stability constant of about $10^{32}$ (Meyer and Abdallah, 1978) than do the siderophores from most microorganisms that are deleterious to plant growth (Scher and Baker, 1982) and their membrane receptors are usually very specific (Hohnadel and Meyer, 1988). Theses features enable the fluorescent pseudomonads to compete efficiently with the soil borne microflora. Pyoverdine-mediated iron competition has been shown to play a determinative role in the microbial antagonism performed by biocontrol strains against some pathogens (Loper and Buyer, 1991; Leong, 1986; Neilands and Leong, 1986; Lemauceau and Alabouvette, 1993).

As discussed above and as shown in Fig 1.11, siderophores may serve as Fe sources or as Fe competitors for organisms, depending on the ability of the organisms to acquire Fe from the stable Fe-siderophore complex. The siderophore mediated interactions as depicted in Fig. 1.11 are for two organisms but may become complicated when more species are present.

**Fig 1.11** Model of siderophore mediated interactions (Buyers and Sikora, 1990). The dashed lines represent ligand exchange by which one Sid displaces another Sid chelating an Fe$^{3+}$ iron. The red lines indicate transport by one organism of the Fe-Sid produced by the other organism. Degradation pathways, including biological and chemical mechanisms, are represented by the black lines and arrows.
Based on this concept it is obvious that if plant growth promoting (PGP) organisms (free-living or symbiotic) used as bioinocula produce siderophore of significant high affinity, it will not only succeed to establish itself, but will also be able to inhibit the growth of pathogenic organisms and hence can act as a biocontrol agent (Chakraborty and Purkayastha, 1984; Ehteshamul-Haque et al., 1992).

1.6 INTRODUCTION TO RHIZOBIA AND THEIR DIVERSITY

1.6.1 What are rhizobia?

Rhizobia are classically defined as symbiotic bacteria capable of eliciting and invading root or stem nodules on leguminous plants, where they differentiate into N$_2$-fixing bacteroids (van Rhijn and Vanderleyden, 1995). Fig. 1.12 shows nodules of leguminous plant after inoculation with *Rhizobium* spp. The symbiotic association between legumes and rhizobia is the most important biocatalytic link for the flow of N between the largest potentially available N reservoir, the atmosphere, and the living world (Paul and Clark, 1989). The *Leguminosae* is the third largest family of flowering plants, with about 650 genera and more than 18000 species (Doyle, 1994; Polhill, 1994). Members of this family are cosmopolitan and ubiquitous, being extremely diverse in their growth habit’s, ranging from tropical canopy trees, lianas and shrubs, to aquatic plants and tiny annual herbs, being dominant species in diverse plant communities.

![Fig 1.12 Nodules of leguminous plant after inoculation with *Rhizobium* spp.](image)

(a) Nodulated roots. (b) Root segment with pink nodules. (c) Longitudinal section showing the structure of a nodule.

*Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea)*

*By cloning and expression of a genomic region encoding iron siderophore receptor*
1.6.2 Rhizobial ecology
A remarkable feature of rhizobial ecology is the ability of these bacteria to change their lifestyles in adaptation to the highly contrasting environments they can inhabit. It has been known for some time that many soils contain a rather large population of non-symbiotic rhizobia that are found both in the bulk soil and in the rhizospheres of legumes and other plants (Segovia et al., 1991; Sullivan et al., 1996; Schloter et al., 1997; Saito et al., 1998). Some of these saprophytic or rhizospheric bacteria may eventually become symbiotic by the horizontal acquisition of a symbiotic plasmid or a chromosomal symbiotic island (Sullivan et al., 1995; Sullivan and Ronson, 1998), allowing them to synthesize and secrete strain-specific lipo-chitooligosaccharides that are strictly required for host nodulation and intracellular invasion (Dénarié et al., 1996; Spaink, 2000). Rhizobia are also found as viable cells in water, where they are able to infect and nodulate aquatic legumes such as Aeschynomene spp. and Sesbania spp. (Chaintreuil et al., 2000; Wang and Martinez-Romero, 2000).

1.6.3 Rhizobial systematics: Phylogenetic analysis of Rhizobia
Rhizobial taxonomy and systematics has progressed notably in the last decade, mainly due to the characterization of new isolates from hosts that had not been previously studied, together with the generalized use of 16S rRNA gene sequencing and polyphasic taxonomic approaches (Martínez-Romero and Caballero-Mellado, 1996; Vandamme et al., 1996; van Berkum and Eardly, 1998). This has led to the description of more than 20 new species and four additional rhizobial genera, i.e. Allorhizobium, Azorhizobium, Mesorhizobium and Sinorhizobium. Based on their 16S rRNA gene sequences, the nodule endosymbionts constitute a polyphyletic assemblage of bacteria grouped in four major phylogenetic branches of the α-2 subclass of the Proteobacteria. Rhizobial strains are currently placed in the following genera: Allorhizobium, Mesorhizobium, Rhizobium and Sinorhizobium, which constitute one of the rhizobial clades; Azorhizobium, Bradyrhizobium, and Methylobacterium, which are each found on a different and well-resolved phylogenetic branch (Sy et al., 2001; Young et al., 2001). These legume microsymbionts are phylogenetically intertwined with several non-symbiotic bacterial genera, comprising
pathogenic, phototrophic and denitrifying strains (van Berkum and Eardly, 1998). Table 1.4 shows the different types of rhizobia and the host plant that they nodulate.

A profound revision of taxonomy of the family \textit{Rhizobiaceae} has recently been proposed, merging the genera \textit{Agrobacterium} and \textit{Allorhizobium} into \textit{Rhizobium}, which reflects the monophylogenetic nature of this clade (Young et al., 2001). The Genera \textit{Mesorhizobium}, \textit{Azorhizobium} and \textit{Bradyrhizobium}, which have traditionally been also included in the family \textit{Rhizobiaceae} (van Berkum and Eardly, 1998), are allocated in the new edition of the Bergey's manual of systematic bacteriology into the new families \textit{Phyllobacteriaceae}, \textit{Azorhizobiaceae} and \textit{Bradyrhizobiaceae}, respectively (the new systematics of rhizobial genera can be found at the URL http://www.cme.msu.edu/bergeys/ page 7). A very interesting recent report is the finding that some highly specific nodule isolates from African \textit{Crotalaria} spp. are phylogenetically placed in a novel 16S rDNA branch of the methylotrophic bacterial genus \textit{Methylobacterium} (Sy et al., 2001). These novel rhizobia were informally assigned to the new species, \textquotedblleft\textit{Methylobacterium nodularis}\textquotedblright, and constitute a newly discovered fourth phylogenetic branch of the rhizobia within the alpha-2 subclass of the \textit{Proteobacteria}. These rhizobia grow facultatively on methanol, which is a characteristic of \textit{Methylobacterium} spp. but a unique feature among rhizobia. Genes encoding two key enzymes of methylotrophy and nodulation, \textit{mxaF} encoding the alpha subunit of the methanol dehydrogenase, and \textit{nodA}, encoding an acyltransferase involved in Nod factor biosynthesis, were detected and sequenced for the \textit{M. nodulans}' type strain ORS2060. Phylogenetic sequence analysis showed that \textit{M. nodulans}' NodA is closely related to \textit{Bradyrhizobium} NodA, suggesting that this gene was acquired by horizontal gene transfer (Sy et al., 2001).

The recent identification of two \textbeta-proteobacterial strains of the genus \textit{Burkholderia} able to nodulate legumes (Moulin et al., 2001) changed the long-held dogma that only bacteria of the \textalpha-subdivision are able to nodulate legumes (van Berkum and Eardly, 1998; Sy et al., 2001). These two strains were subsequently described as \textit{Burkholderia tuberum} and \textit{Burkholderia phymatum} (Vandamme et al., 2002). In addition, eight strains isolated from root nodules of \textit{Mimosa} spp. were recently described as \textit{Ralstonia taiwanensis}, also
classified as β-proteobacteria (Chen et al., 2001), although their nodulation capacity was not confirmed. The terms α- and β-rhizobia were proposed to distinguish the rhizobial α- and β-proteobacteria, respectively (Moulin et al., 2001).

The above summarized findings lend strong support to the notion that the huge diversity of leguminous plants is paralleled by a large diversity of rhizobial microsymbionts as shown in Fig 1.13 (Martínez-Romero and Caballero-Mellado, 1996).
Chapter 1: Review of Literature

Meikylobacterium nodulans Gen Bank AF220763
B. japonicum strain IAM 12608 Gen Bank D12781
B. japonicum Gen Bank U59638
B. elkanii Gen Bank U35000
Allorhizobium undicola Gen Bank Y17047
Rhizobium mongolense Gen Bank U89822
Rhizobium gallicum Gen Bank U86343
Rhizobium leguminosarum Gen Bank U29386
Rhizobium etli Gen Bank U28916
Rhizobium sp. RCR3613D Gen Bank U29387
Agrobacterium rhizogenes Gen Bank X67224
Rhizobium tropici Gen Bank U89832
Mesorhizobium sp. HLS6 Gen Bank AF041445
Mesorhizobium tianshanense Gen Bank AF041447
Rhizobium ciceri Gen Bank U07934
Mesorhizobium loti Gen Bank X67229
Rhizobium loti Gen Bank U50165
Rhizobium mediterraneum Gen Bank L38825
Mesorhizobium huakuii Gen Bank D13431
Sinorhizobium melliloti Gen Bank D14509
Sinorhizobium fredii Gen Bank X67231
Sinorhizobium saheli Gen Bank X68390
Sinorhizobium terangae Gen Bank X68387

Fig 1.13 Phylogenetic tree showing the clustering of members of rhizobia.

Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea)
By cloning and expression of a genomic region encoding iron siderophore receptor
Table 1.4 List of different rhizobial genera and the plants they nodulate

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alorhizobium</em></td>
<td><em>A. undicola</em></td>
<td><em>Neptunia natans</em></td>
</tr>
<tr>
<td><em>Azorhizobium</em></td>
<td><em>Az. caulinodans</em></td>
<td><em>Sesbania rostrata</em></td>
</tr>
<tr>
<td><em>Bradyrhizobium</em></td>
<td><em>B. elkanii</em></td>
<td><em>Glycine max</em></td>
</tr>
<tr>
<td></td>
<td><em>B. japonicum</em></td>
<td><em>G. max</em></td>
</tr>
<tr>
<td></td>
<td><em>B. liaoningense</em></td>
<td><em>G. max</em></td>
</tr>
<tr>
<td><em>Mesorhizobium</em></td>
<td><em>M. anorphae</em></td>
<td><em>Amorpha fruticosa</em></td>
</tr>
<tr>
<td></td>
<td><em>M. chacoense</em></td>
<td><em>Prosopis alba</em></td>
</tr>
<tr>
<td></td>
<td><em>M. ciceri</em></td>
<td><em>Cicer arietinum</em></td>
</tr>
<tr>
<td></td>
<td><em>M. hiakutii</em></td>
<td><em>Astragalus</em></td>
</tr>
<tr>
<td></td>
<td><em>M. loti</em></td>
<td><em>Loti</em></td>
</tr>
<tr>
<td></td>
<td><em>M. mediterraneum</em></td>
<td><em>Cicer arietinum</em></td>
</tr>
<tr>
<td></td>
<td><em>M. pluriflorum</em></td>
<td><em>Acacia, Leucaena</em></td>
</tr>
<tr>
<td><em>Methylobacterium</em></td>
<td><em>M. nodulans</em></td>
<td><em>Crotalaria pedocarpa</em></td>
</tr>
<tr>
<td><em>Rhizobium</em></td>
<td><em>R. etli</em></td>
<td><em>Phaseolus vulgaris</em></td>
</tr>
<tr>
<td></td>
<td><em>R. galegae</em></td>
<td><em>Galega</em></td>
</tr>
<tr>
<td></td>
<td><em>R. gallicum</em></td>
<td><em>P. vulgaris</em></td>
</tr>
<tr>
<td></td>
<td><em>R. giardinii</em></td>
<td><em>P. vulgaris</em></td>
</tr>
<tr>
<td></td>
<td><em>R. hainanense</em></td>
<td><em>Centrosema, Desmodium, Stylosanthes, Tephrosia</em></td>
</tr>
<tr>
<td></td>
<td><em>R. huautense</em></td>
<td><em>Sesbania herbacea</em></td>
</tr>
<tr>
<td></td>
<td><em>R. leguminosarum</em></td>
<td><em>Trifolium, Vicia</em></td>
</tr>
<tr>
<td></td>
<td><em>R. mongolense</em></td>
<td><em>Medicago ruthenica</em></td>
</tr>
<tr>
<td></td>
<td><em>R. phaseoli</em></td>
<td><em>P. vulgaris</em></td>
</tr>
<tr>
<td></td>
<td><em>R. trifolii</em></td>
<td><em>Trifolium</em></td>
</tr>
<tr>
<td></td>
<td><em>R. yanglingense</em></td>
<td><em>Amphicarpaea, Triesperma Carollina varia and Gueldenstaedtia multiflora</em></td>
</tr>
<tr>
<td><em>Sinorhizobium</em></td>
<td><em>S. arboris</em></td>
<td><em>Acacia senegal</em></td>
</tr>
<tr>
<td></td>
<td><em>S. fredii</em></td>
<td><em>Prosopis chilenensis</em></td>
</tr>
<tr>
<td></td>
<td><em>S. kostiense</em></td>
<td><em>G. max</em></td>
</tr>
<tr>
<td></td>
<td><em>S. medicae</em></td>
<td><em>A. senegal, P. chilenensis</em></td>
</tr>
<tr>
<td></td>
<td><em>S. maliori</em></td>
<td><em>Medicago sativa</em></td>
</tr>
<tr>
<td></td>
<td><em>S. saheili</em></td>
<td><em>Sesbania</em></td>
</tr>
<tr>
<td></td>
<td><em>S. terangae</em></td>
<td><em>Acacia, Sesbania</em></td>
</tr>
<tr>
<td></td>
<td><em>S. xinjiangense</em></td>
<td><em>G. max</em></td>
</tr>
</tbody>
</table>

Construction of a transgenic *Rhizobium* for Cajanus cajan (pigeon pea) by cloning and expression of a genomic region encoding iron siderophore receptor
1.7 IRON METABOLISM IN RHIZOBIA

Nitrogen-fixing rhizobia, living as endosymbionts in root nodules of legume host plants, have a high demand for iron because a number of 'symbiotic' proteins contain iron or heme (Guerinot, 1993). For example, the nitrogenase complex that makes up more than 10% of the total cellular protein of nitrogen-fixing bacteroids contains 34 iron atoms per molecule. Similarly, several different types of cytochromes are synthesized at high levels in bacteroids. Despite the importance of iron in these organisms, iron metabolism is relatively poorly studied in most rhizobial species.

1.7.1. Siderophore production in Rhizobia

Diverse types of siderophores are produced by the different rhizobial genera e.g. *Rhizobium leguminosarum* bv. *viciae*, the symbiont of peas, lentils, vetches and some beans, synthesizes a cyclic trihydroxamate type siderophore called *vicibactin* which is taken up by cells via outer and inner membrane machinery encoded by *fkuABCD* operon (Dilworth et al., 1998). *Rhizobium meliloti* DM4 produces rhizobactin in which ethylenediamine is the iron chelator (Smith et al., 1985), whereas *Sinorhizobium meliloti* 2011 under iron stress, produces *rhizobactin 1021* a dihydroxamate siderophore (Persmark et al., 1993) and the genes *rhbABCDEF* responsible for its biosynthesis were cloned (Gill and Neilands, 1989) and characterized (Lynch et al., 2001). *Catecholate siderophores* are known to be produced by rhizobia from the cowpea group (Jadhav and Desai, 1992; Modi et al., 1985) and catecholates viz., *salicylic acid* and *dihydroxybenzoic acid* are produced by *Rhizobium ciceri* isolates from chick pea nodules (Berraho et al., 1997). *Citrate* as a siderophore is released by *Bradyrhizobium japonicum* under iron-limited conditions (Guerinot et al., 1990). Anthranilate is siderophore produced by *Rhizobium leguminosarum*. Table 1.5 shows the list of few rhizobial siderophores.
### Table 1.5: List of rhizobial siderophores (Carson, et al., 2000)

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of Plant</th>
<th>Component(s)</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alfalfa</td>
<td><em>R. meliloti</em> 2011</td>
<td>Rhizobactin 1021</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>R. meliloti</em> DM4</td>
<td>Carboxylate type</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>R. meliloti</em> 1021</td>
<td>Dihydroxylate type</td>
</tr>
<tr>
<td>2</td>
<td>Pea</td>
<td><em>R. leguminosarum</em> biovar viciae</td>
<td>Cyclic trihydroxamate type, vicibactin</td>
</tr>
<tr>
<td>3</td>
<td>Cowpea</td>
<td>Cowpea <em>Rhizobium</em></td>
<td>Catecholate</td>
</tr>
<tr>
<td>4</td>
<td>Soybean</td>
<td><em>Bradyrhizobium japonicum</em> 61A152</td>
<td>Citrate type</td>
</tr>
</tbody>
</table>

### 1.7.2 Siderophore Uptake Machinery and its regulation in Rhizobia:

Although siderophore uptake machinery of rhizobia is poorly characterized, it can be said that this group of microorganisms have similar system of siderophore mediated uptake machinery as other gram negative species.

### 1.7.2.1 IROMPs in Rhizobia

Relatively few reports describe the presence of Iron Regulated Outer Membrane Proteins (IROMPs) in rhizobia that are produced and bind with their specific siderophore iron complex (Reigh and O’Connell, 1993; Jadhav and Desai, 1994; Patel et al., 1994).

Stevens et al., (1999) identified some of the *fhu* genes of *R. leguminosarum* which are homologues of the hydroxamate siderophores uptake machinery in *E. coli*. One of them is FhuA that specifies the OM receptor for uptake of vicibactin and works in association with FhuCDB (inner membrane proteins). FhuC is the ABC-transporter ATPase, FhuB is the permease and FhuD is the periplasmic siderophore binding protein which brings the ferri-vicibactin complex to the inner membrane machinery for its transport from the periplasm to the cytoplasm (Braun and Braun, 2002). Stevens *et al.* (1999) also identified a pseudogene version of *fhuA*, next to the functional *fhuCDB* genes and pseudogene versions of *FhuA* have also been detected in several other strains of *R. leguminosarum* (Yeoman et al., 2000).
Another receptor is rhizobial ferrichrome OM receptor FegA of *B. japonicum* 61A152. It is a hydroxamate-type siderophore receptor (LeVier and Guerinot, 1996). The fegA gene is organized in an operon with fegB which probably encodes an inner membrane protein (Benson et al., 2005). Mutant analysis revealed that both genes are required for utilization of the siderophore ferrichrom. FegA has been shown to bind ferrichrome specifically and its homologs have been detected in other strains as well (LeVier and Guerinot, 1996). RhtA is an OM receptor responsible for rhizobactin uptake in *Sinorhizobium meliloti*, where a specialized single permease RhtX is responsible for its transport from periplasm to cytoplasm (O'Cuiv et al., 2004).

Noya et al., (1997) reported that several rhizobial species, including *B. japonicum*, are able to grow on haemoglobin, or haem, as sole iron source, a property hitherto described exclusively for pathogenic bacteria (Genco and Dixon, 2001). This observation suggested the existence of haem uptake systems in these rhizobia which was confirmed in *B. japonicum* (Nienaber et al., 2001) as well as *R. leguminosarum* (Wexler et al., 2001) HmuR is the heme receptor in *Bradyrhizobium japonicum* (Nienaber et al., 2001). Homologues of HmuR and HmuTUV are encoded in the genomes of *Sinorhizobium meliloti* ([http://sequencetoulouse.inra.fr/meliloti.html](http://sequencetoulouse.inra.fr/meliloti.html)) and *Mesorhizobium loti* ([http://www.kazusa.or.jp/rhizobase/](http://www.kazusa.or.jp/rhizobase/)).

LeVier and Guerinot (1996) have compared the amino acid similarities and identities among rhizobial receptor FegA with other non-rhizobial related siderophore receptor proteins. They have shown that FegA is 53.7% similar to the *E. coli* Fe (III)-ferrichrome receptor FhuA and 48.3% similar to coprogen and rhodotorulic acid receptor FhuE.

Like any other ferrisiderophore uptake machinery the rhizobial high affinity Fe-uptake system also requires other components other than the receptor for the transport of the ferrisiderophore-complex. Wexler et al, (2001) had shown *tonB*-like gene (*tonB*), adjacent to an operon that specified an ABC transporter involved in haem uptake in *R. leguminosarum*. He also showed that the two of the *tonB* mutants, J350 and J344, had large halos on CAS plates due to higher amounts of the siderophore vicibactin that got
accumulated in the extracellular medium. This was probably because the tonBR\! mutants failed to import vicibactin and thus indicated that TonB\! was required for the Fhu transport system to operate, just as it was found in other bacterial siderophore uptake systems. Whereas Nienaber et al, (2001) has shown by mutant analysis and complementation tests that the TonB system was specific for haem uptake and was dispensable for siderophore uptake proposing the existence of a second TonB homologue functioning in the uptake of Fe-chelates in Bradyrhizobium japonicum.

1.7.2.2 Regulation of ferri-siderophore assimilation system in Rhizobia:

Fur mediated regulation which is highly conserved among most bacterial species is also present in Bradyrhizobium japonicum (Hamza et al., 1999) and Rhizobium leguminosarum (De Luca and Johnston, 1998) since fur genes have been identified in these species. Yang et al, (2006) findings show that B. japonicum Fur proteins have a novel function in the iron-dependent gene expression. The B. japonicum Fur protein has been shown to regulate the hemA gene (Hamza et al., 1998).

Another member of the Fur family is Irr (iron response regulator), which occurs in rhizobia. Irr was first identified in B. japonicum, as a transcriptional repressor of hemB, which specifies 8-aminolaevulinic acid dehydratase, in the haem biosynthetic pathway (Hamza et al., 1998). Irr is restricted to a few α-proteobacteria including rhizobia, Agrobacterium, the animal pathogen Brucella and Rhodopseudomonas palustris a photosynthetic bacterium very closely related to B. japonicum. Hamza et al (1999) showed that the transcription of irr was moderately repressed in Fe-replete conditions, and this was shown to be dependent on a protein FurB\! that was homologous to Fur even though the irr promoter region had no sequence similarity to the canonical fur boxes (Hamza et al., 2000). These observations suggest that rhizobial Fur differs from that of other bacteria including E. coli. Recently Yang et al, (2006b), had shown the control of the expression of iron transport genes and many other iron-regulated genes which are not directly involved in haem synthesis in Bradyrhizobium japonicum through Irr. Irr was shown to have both a positive and negative effect on the gene expression. Their findings indicated that B. japonicum sensed iron via the
status of haem biosynthesis in an Irr-dependent manner, and thus regulated iron homeostasis and metabolism.

Regulation of iron responsive genes in *R. leguminosarum* and *S. meliloti* is not mediated by Fur but rather by the dissimilar RirA (rhizobial iron regulator) protein a member of the Rrf2 family, is a newly found gene whose product has no sequence similarity to Fur but which has close homologues in other rhizobia, like *Agrobacterium* and *Brucella* (Todd et al., 2002). All the above reports state that the regulation of Fe-responsive genes in rhizobia is not mediated by Fur alone.

There is also positive regulation reported in rhizobia. In the heme uptake system of *B. japonicum* encoded by the gene cluster hmuVUT-hmuR-exbBD-tonB, transcription of the divergently oriented hmuT and hmuR genes was not only found to be induced by iron limitation but to also depend on a 21-bp promoter-upstream iron control element (ICE) which by deletion analysis was shown to be needed for positive control (Rudolf et al., 2006).

### 1.7.3 Importance of iron acquisition in rhizobial-legume symbiosis

There is a high demand for iron in the symbiotic interaction between legumes and rhizobia, although the mechanism by which it is provided in plant root nodules is not well understood. Recently Stacey et al., (2006) has studied the functional genome analysis of legume nodulation. A nodulated legume has an increased need for iron compared to a non-nodulated plant (Guerinot, 1991; Deryto and Skorupska, 1992) since this metal is a constituent of key proteins such as nitrogenase and leghaemoglobin. Nitrogenase is made up of two proteins; both are rich in iron and essential for activity. Non-heme iron electron transferproteins such as ferredoxin and flavodoxin are essential in nitrogen fixation (Eady and Postgate, 1974). Iron deficiency in nodulated legumes is very common on alkaline soils, and affects such common agricultural crops as chick pea (Rai et al., 1982), French bean (Hemantaranjan and Garg, 1986) and peanut (O’Hara et al., 1988). The importance of iron in the legume-rhizobia symbiosis was demonstrated by O’Hara et al. (1988), who showed that iron deficiency limits nodule development but not initiation in peanuts inoculated with *Bradyrhizobium* species. Nodulation is also drastically curtailed in lupines under iron deficiency (Tang et al., 1990).

*Construction of a transgenic Rhizobium for Cajanus cajan (pigeon pea) By cloning and expression of a genomic region encoding iron siderophore receptor*
Specific siderophore producing microorganisms stimulated the nodulation, nitrogen fixation and plant growth of leguminous plants (Grimes and Mount, 1987; Omar & Abd-Alla, 1994; Shenker et al., 1999). The authors suggested that, nodulation promoting microorganisms could be included in rhizobial inoculants to improve efficacy of these products. The mechanisms of action under field and laboratory conditions are still under investigation. One of the possible modes of growth promotion of nodulated legumes under field conditions is production of siderophores which control the proliferation of soil-born pathogens or facilitate the uptake of iron from environment (Schippers et al., 1987; Omar and Abd-Alla, 1998). However, taking into consideration that iron stressed plants show fewer bacteroids present in the nodules, decreased amount of leghemoglobin and lower specific nitrogenase activity and that possession of the ability to produce siderophore significantly increases the efficiency of the differentiated bacterium to fix nitrogen and induce an increase in plant growth, 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., 1988b).

Mutants of *R. meliloti* 1021 defective in the production of rhizobactin 1021 have been isolated and tested for their nodulation ability and efficiency in nitrogen fixation. Since effective nodules were formed containing viable bacteria it was concluded that the rhizobial iron assimilation system served no obvious role in nodulation and bacteroid development (Gill and Neilands, 1989). Nadler et al. (1990) showed that *Rhizobium leguminosarum* 116, an ineffective mutant strain with a defect in iron acquisition, formed white ineffective nodules on peas and had an apparent defect in iron acquisition. Gill et al. (1991) investigated the nitrogenase activity of plants inoculated with wild-type and rhizobactin 1021 mutants over a 30-day period and found a minor increase in activity in the plants inoculated with the wild type. In a second experiment over a 70-day period, a significant increase in nitrogenase activity and total plant dry weight was observed with the wild type compared to some rhizobactin 1021 mutants. Barton et al. (1992) also observed increased efficiency in nitrogen fixation by plants growing in low iron medium when inoculated with the wild type compared to plants inoculated with rhizobactin 1021 mutants. These studies suggest that rhizobactin 1021, while not essential for symbiosis, can contribute to the efficiency of
nitrogen fixation under certain conditions of plant growth. Other studies have shown conflicting results: in some cases, rhizobial mutants defective in siderophore synthesis fix $N_2$ normally (Reigh and O'Connell, 1993), but in others, Sid$^{-}$ mutants fail to fix $N_2$ symbiotically (Barsomian et al., 1992). In a later study it was shown that, in iron-rich medium, the regulation of rhizobactin synthesis is a factor in efficient nitrogen fixation (Barton et al., 1996).

With the aim of elucidating the implication of iron and iron transport mechanisms in the three-partner interaction rhizobia legume soil, Fabiano et al. (1994) evaluated the ability to express high-affinity iron transport systems in an Uruguayan collection of rhizobia isolated from nodules of native, naturalized and introduced legumes. Results obtained indicated that iron acquisition systems are widespread in Uruguayan isolates. Research performed by Fabiano et al. (1995), with the native strain $S. \text{meliloti}$ 242 demonstrated that siderophore-mediated iron uptake systems are not essential for an efficient biological nitrogen fixation but they are involved in early steps of nodulation and in rhizobia competitiveness (Platero et al., 1999).

Besides mutants affected in siderophore biosynthesis, mutants affected in other aspects of iron uptake and regulation also gave similar confusing findings. A fur-null mutant of $B. \text{japonicum}$, shown to be derepressed for iron uptake in culture formed an effective symbiosis (Hamza et al., 1999), whereas a manganese-resistant fur mutant strain (Benson et al., 2004) was unable to form functional, nitrogen-fixing nodules on soybean, mung bean, or cowpea, suggesting possible roles for a Fur-regulated protein or proteins in the symbiosis at least under some circumstances. Yeoman et al (2000) isolated a mutant $\text{Rhizobium leguminosarum}$ for gene $fhuA$, which specified the outer-membrane receptor for the uptake of siderophore vicibactin. The mutant was defective in iron uptake and accumulated the siderophore vicibactin but did not detectably affect symbiotic $N_2$ fixation on peas. Also $fhuA::gus$ fusion was expressed by bacteria in the meristematic zone of pea nodules, but not in mature bacteroids suggesting that vicibactin is not used for iron uptake in $R. \text{leguminosarum}$ bacteroids. Benson et al (2005) showed that both $fegA$ and $fegB$ were required for the utilization of ferrichrome, where as $fegB$ mutant was found to form a normal

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symbiosis, the fegAB mutant had a dramatic phenotype in planta, speculated by them to be due to the inability of the mutant to transport ferrichrome. This symbiotic defect suggested that the fegAB operon was serving a different function in planta, possibly thought to be involved in the signalling between the rhizobia and the host plant. It is generally believed that siderophore biosynthesis or transport genes are not expressed when nitrogenase genes are actively expressed. How the supply of iron is provided to form nitrogenase, at a stage in nodulation when it is highly active, remains to be resolved. It may be possible that iron nutrition of the bacteroids in the nodules is through ferrous form of iron which becomes available to the bacteroids through the reductases present on the peribacteroid membrane (Moreau et al., 1998). It has also been suggested that there could be additional, unknown bacteroid-specific siderophore system. Indeed recently, Ampe et al (2003), while studying the expression of 200 genes of S. meliloti upon induction with plant symbiotic elicitor luteolin and during symbiosis, found three genes related to iron metabolism were induced by luteolin, and also in nodules. One codes for a probable siderophore, sitA for an iron transporter and third is homologous with a gene of the tonB-hmu cluster of R. leguminosarum. SitABC-transporter thus seems to be an important system for iron acquisition in planta.

1.8 RHIZOBIAL COMPETITION FOR IRON

Siderophore production and cross-utilization has good implications for free-living rhizobia of being able to survive better in the soil as compared to siderophore non-producing strains.

1.8.1 Competition affecting rhizospheric colonization

For members of the genus Rhizobium to induce nodulation on their leguminous hosts, the bacteria probably must grow in the rhizosphere as well as at the site on the root hair where invasion of the plant begins. In these environments, the rhizobia are probably growing at the expense of organic products excreted by the roots, but these bacteria must compete for the essential nutrients (like iron) with other heterotrophic inhabitants of the rhizosphere. Many rhizobial strains have the potential to increase plant growth and yields, but are poor at nodulation due to weak rhizospheric colonization. In 1941, Nicol and Thornton studied the role of competition among strains of Rhizobium spp. for nodule sites and the impact of such
intraspecific competition on nodulation. Their work dealt with the behaviour of mixed strains of nodule bacteria towards each other and towards their legume host, where they showed that when two strains of nodule bacteria were present in the surroundings of their host's root system, active competition between them caused the strain having a higher initial growth rate almost to completely check the multiplication of the other strain outside the plant. This dominant strain was then responsible for nearly all the nodules produced in the plant.

In the rhizosphere, however, the dominant species are not members of the genus *Rhizobium*, and because the dominant bacteria in that environment are often able to grow rapidly (Rouatt and Katznelson, 1957), they are probably able to compete effectively with *Rhizobium* spp. and to keep the rhizobial population small. The finding that *R. trifolii*, *R. phaseoli*, and *B. japonicum* multiplied in soil when other bacteria were suppressed, but not when other genera were not inhibited suggests that competition for the supply of available nutrients is important, at least in soil apart from the roots (Pena-Cabriales and Alexander, 1983). Such intergeneric competition has scarcely been explored. Hely et al. (1957) suggested that the native microbial communities of certain Australian soils prevented the nodulation of *Trifolium subterraneum* because these microorganisms suppressed the colonization of the plant roots by *R. trifolii*. Several investigators have demonstrated that individual isolates of bacteria or fungi decrease the nodulation of aseptically grown subterranean and white clovers by *R. trifolii* (Harris, 1953; Anderson, 1957; Plazinski and Rolfe, 1985) or the rate and extent of nodulation of aseptically grown alfalfa by *R. meliloti* (Handelsman and Brill, 1985.). In two of these studies, it was shown that the organisms causing the suppression did not produce toxins against *R. trifolii* in culture (Anderson, 1957; Plazinski and Rolfe, 1985), indicating that the suppression of nodule formation resulted from competition with the root nodule bacteria; nevertheless, the inhibition could have resulted from a microbiologically induced change in the physiology of the host. De-Ming Li and Alexander (1986) showed that growth rates greatly affected the outcome of competition between *R. meliloti* and other bacteria. Bacteria with slow growth rates did not significantly affect *R. meliloti*, whereas those that multiplied rapidly were good competitors. Bacteria that
grew faster than *R. meliloti* reduced the number of nodules, and those that grew more slowly had no such effect.

There is little evidence that iron deficient soils affect the numbers of root nodule bacteria. In iron stressed soils, the proportion of siderophore-producing strains appear to increase. In alkaline soils of the mid-western United States *B. japonicum* serotype 135, a siderophore producer, has been shown to dominate over serotype 123, a siderophore non-producer (Manjanatha et al., 1992). Similarly, 54% of *S. meliloti* strains isolated from alfalfa nodules from alkaline soil were siderophore producers whereas from only 18% siderophore producing strains were obtained from an acidic soil (Barran and Bromfield, 1993). These reports do suggest the possible advantage of siderophore production in iron deficient soil in the increased survivability and number of the producing strain in the rhizosphere.

1.8.2 Heterologous siderophore utilization a sound strategy to outcompete

Complexation of Fe(III) by soil anions as well as competition for Fe(III) with other soil microorganisms are obstacles to iron acquisition commonly faced by *Rhizobium* spp. and all soil microflora. Rhizobia must be able to persist in the soil in the absence of their host plants and competition for iron, the availability of which is limited by its insolubility, is one of the factors in determining how successful rhizobia are in maintaining themselves in the rhizosphere (Guerinot, 1991). The rhizobial high affinity iron uptake systems may be of use in the competition among soil microbes for access to available iron and may enhance the survival of the free-living forms. Evidence has been presented to indicate that soil competition among rhizosphere pseudomonads may occur at the level of the uptake system of the ferric-complex specific for the individual *Pseudomonas* sp. siderophore (Buyer and Leong, 1986). It has been shown that *R. meliloti* DM4 excretes and utilizes the siderophore rhizobactin, whereas several other wild-type *R. meliloti* strains do not (Smith and Neilands, 1984) thus it is possible that a comparable competition might also occur among rhizobial strains. Therefore a rhizobial strain having the ability to utilize siderophore(s) produced by another similar rhizobial strain(s) [homologous siderophore] or from a non-rhizobial strain [heterologous siderophore] should also have a competitive advantage in the rhizosphere. It could be therefore said that possession of uptake system for the siderophore produced by

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majority organisms in the soil, and hence predominantly present in the soil can have positive implications for growth and survival of the possessing organism.

Certain rhizobia have also been shown to utilize iron bound to heterologous siderophores. *Bradyrhizobium japonicum* USDA110 and 61A152 can utilize ferrichrome (made by numerous fungi), rhodotorulate (made by yeast), pseudobactin (made by pseudomonads) etc (Plessner et al., 1993) and these strains are successfully used as commercial bioinocula for soybeans. *R. meliloti* DM4 can utilize the hydroxamate type siderophores, ferrichrome and ferrioxamine B (made by actinomyces) in addition to its own siderophore rhizobactin (Smith and Neilands, 1984).

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1.9 Present Investigation:
Availability of iron has been a major problem for microorganisms ever since the emergence of an aerobic/facultative lifestyle and siderophore-dependent iron acquisition systems has been the evolutionary answer to this problem. As discussed in the earlier section, ferrisiderophore uptake systems also mediate a variety of ecologically important interactions among different species and a deeper understanding of these could help predict the fate of bacteria such as rhizobia which are used as bioinoculants to improve plant growth. In order to efficiently nodulate the host plant, specific rhizobial strains which are applied as biofertilizers should be able to survive and multiply in free-living state in the soil and face competition from native rhizobia preexisting in the niche as also other species in the rhizosphere. Efficiency of iron uptake system in rhizobia is expected to be one of the decisive factors in effective nodulation and establishment of symbiotic relationship of this organism with the host.

This work deals with the study of siderophore-mediated interactions among rhizobia using pigeon pea (Cajanus cajan) and its nodule bacteria as the focus of study. An aspect of this work was to establish the feasibility improving iron nutrition of this group of bacteria by heterologous expression of gene(s) imparting the ability to utilize additional siderophore complexes. Thus the aim of this research was to throw light on the role that rhizobial siderophores play in competition with each other. The research also focuses on the isolation of nodule assisting rhizobacteria with potential to improve rhizobial growth and nodule forming ability and characterization of role of siderophores in such interactions.

1.9.1 Broad objective of the present investigation:
Study of siderophore mediated rhizobial interactions and the effect of improved siderophore utilizing ability of rhizobial biofertilizer strains on their growth and survival.

1.9.2 Work plan for present investigation:
1. Isolation and characterization of siderophore producing C. cajan nodule isolates.
2. Study of ability of different strains to cross-utilize heterologous siderophores.

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3. To study whether good siderophore cross-utilizing ability supports advantage in growth and survivability of the isolate.

4. To clone and express the *E. coli* ferrichrome receptor gene (*fhuA*) into rhizobial biofertilizer strains.

5. Effect of the *fhuA* gene on the growth of rhizobia and its ability to improve plant health with respect to the parent strain.

6. Isolation and characterization of nodule assisting rhizobacteria (NAR) of *C. cajan* and its effect on rhizobial growth and in turn on the plant health.