Analysis of Siderophore production by Rhizobium meliloti 220-5.

Thesis

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I hereby declare that the research described within this thesis is based entirely upon my own work.

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DEDICATED TO MY PARENTS

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" Gold is for the mistress, Silver for the maid. Copper for the craftsman cunning at his trade ! Good ! Said the Baron, sitting in his hall, But Iron -cold Iron- is master of them all."

Rudyard Kipling.

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ABSTRACT

A universal chemical assay (Schwyn and Neilands, 1987) was used to detect the production of siderophore in a range of rhizobacterial strains. Siderophore production was found to be strain specific in *Rhizobium*. The production of siderophore by *Rhizobium meliloti* 220-5 was examined in detail. Using the universal assay to test samples taken during growth of the bacterium in an iron deficient medium, it was established that production of the siderophore commenced at the onset of exponential growth.

The cell free supernatant of *R.meliloti* 220-5 grown under iron deficient conditions and shown to contain a siderophore, was tested for the presence of catechol or hydroxamate groups. Neither group was detected.

Using a phenol/chloroform extraction procedure the siderophore was isolated from low iron supernatants and purified by ion-exchange chromatography. IR spectra confirmed that the siderophore was neither catechol nor hydroxamate in nature. This indicates that it belongs to a third class of siderophores typified by rhizobactin from *R.meliloti* DM4 (Smith and Neilands, 1984).

Mutants of R.meliloti 220-5 and R.meliloti 2011 defective in the production of siderophore were isolated following transposon mutagenesis with Tn5-mob. The genomic sequence containing the transposon from R.meliloti 220-5-1 was cloned into pUC19 and used to screen a cosmid bank of R.meliloti 2011. Two cosmids hybridising were investigated. One cosmid, cosmid 3, complemented the mutation in R. meliloti 220-5-1. When the other mutants were tested for complementation by cosmid 3, the complementation pattern indicated that cosmid 3 harbours more than one gene involved in the siderophore biosynthesis. The number of siderophore genes carried on this cosmid remains to be determined.

SDS-polyacrylamide gel electrophoresis of outer membrane proteins prepared from iron deficient cultures identified the presence, in *R.meliloti* 220-5, of two low iron induced proteins of 72,000 and 78,000 daltons. These induced proteins were cut from preparative SDS-PAGE gels and used as antigens to raise polyclonal antibodies. Serum collected from the injected rat was tested for the antibody by Western blot analysis. Chapter one Introduction.

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1.1 General Introduction.

Most microorganisms have an absolute nutritional requirement for iron. It is the fourth most abundant metal in nature but this abundance is essentially negated by its insolubility in an aerobic environment. In aqueous medium at neutral pH, iron exists as an insoluble polymer, (FeOH₃), having a solubility constant of 10^{-38} (Biederman and Schindler, 1957). At pH 7.4, generally taken as representing biological pH, the solubility of free iron is 10^{-18} , a value too low to support microbial growth.

To combat the low solubility of external iron, those bacterial and fungal species living all or part of their time in an aerobic environment have evolved low molecular weight carriers generically termed siderophores for the acquisition of iron from the soil, marine or fresh water environments and from animal host tissues (Neilands,1972; Lankford, 1973).These compounds are marked by an extreme affinity and therefore specificity, for the trivalent oxidation state of iron. Their efficient absorbtion at low concentration is achieved by receptors in the cell envelope.

The siderophore system of receptor dependent, high affinity iron assimilation is widely distributed in the microbial world and is found in gram-negative and gram-positive bacterial species, including animal and plant pathogens, in fungi and in cyanobacteria. A few species, apart from the anaerobes, appear not to form a siderophore system. Among bacteria this includes certain lactobacilli, *Legionella* and *Neisseria* and among the fungi, *Saccharomyces cerevisiae* (Reeves 1983).

In a particular bacterial strain or species, elimination, by genetic means, of the siderophore-mediated high affinity uptake system only becomes growth limiting under conditions where iron is severely restricted. Lacking chelates and receptors for genetic manipulation, there is nothing known at this time about how the low affinity iron pathway functions. It can be suggested that a cell could form reducing agents since ascorbic acid will substitute for enterobaction (a siderophore produced by E.coli) in enterobactin mutants (Pollack *et al.*, 1970 a).

Efficient, non-siderophore systems may exist whereby iron is taken in by a process parallel to the endocytosis route of the, eucaryotic world. Thus in microorganisms the uptake pathways for environmental iron appear to exhibit considerable variation.

1.2 Siderophores and their molecular structures.

Within the siderophore system of iron uptake, the ligands produced by iron stressed microorganisms also exhibit considerable variation, unlike enzymes and other macromolecules which remain almost constant throughout а range of microorganisms. Despite this variation siderophores can be divided into two main structural groups; the hydroxamates, which are produced by both fungi and bacteria, and the phenolates/catecholates, which are usually found in bacteria.

1.2.1 The Hydroxamates.

Most hydroxamate siderophores contain three secondary hydroxamate groups. Each hydroxamate group provides two oxygens which form a bidentate ligand with iron, resulting in a hexadentate octahedral complex with Fe³⁺ for each siderophore. Ferrichromes, one example of hydroxamate siderophores, are cyclic hexapeptides containing a tripeptide of glycine, alanine or serine and a tripeptide of N^{δ}-acyl-N^{δ}-hydroxyornithine. The core structure is shown in Fig. 1.1.



The choice of three neutral amino acids (R_1, R_2, R_3) in the cyclohexapeptide structure and the fact that the acyl substituent, (R), on the δ -nitrogen of hydroxylamino ornithine may be any one of a number of small carboxylic acids, likely

number of variants of the structure shown.

Ferrichrome, (figl.2a), produced by the smut fungus Ustilago sphaerogena, isolated by Neilands in 1952, was the first trihydroxamate identified in nature and is the protypical type hydroxamate siderophore. It is one of a few siderophores, the x-ray structure of which has been determined (van der Helm et 1980). Malonichrome (Emery, 1980). carrying three al., molecules of malonic acid as the acyl substituent and produced by prolonged culture of Fusarium roseum is an example of a ferrichrome type siderophore. Albomycin, (figl.2b), also a close derivative of ferrichrome, is produced by Streptomyces and has antibiotic activity.

The ferrioxamines are a group of siderophores generally produced by the Actinomycetes. These compounds are linear or trihydroxamates built from structural cyclic units of 1-amino-w-hydroxylaminoalkane and succinic or acetic acid (Bickel et *al.*,1960).The methane sulphonate salt of deferriferrioxamine B (i.e. the deferrated form, figl.2c), is commercially available from Ciba-Geigy as Desferal and is the drug most commonly used in the treatment of iron overload. The ferrioxamines also include the antibiotic ferrimycin.

Fusarinines, the fungal siderophores, have the basic structure shown.



They contain this naturally occuring monohydroxamic acid joined by ester groups rather than peptide bonds. The functional siderophore is believed to be the cyclic trimer of the unit shown, fusarinine C or fusigen. (Figl.2d).

Fusarinines are produced by Fusarium and Penicillium spp..., Aspergillus nidulans and Penicillium chrysogenum excrete fusarinine C as an extracellular siderophore. Ferric N,N',N'triacetyl fusarinine C (triacetyl fusigen) reported from a number of fungi, is more stable than the non-acylated parent compound and later during the growth cycle this more stable but less active form is produced by A.nidulans (Moore et al., 1976). Penicillium sp. can also produce the triacetylated derivative at a concentration of $300 \text{mg} \text{ l}^{-1}$. It is thought that iron complexed by the triacylated siderophore is withheld from competing microorganisms but can be transferred to the active siderophore when needed by the producing organism. The ester linkages in fusarinines are very susceptible to hydrolysis and the linear, trimer, dimer and monomer chains are frequently observed in the culture filtrate along with the cyclic trimer (Sayer and Emery, 1968; Charlang et al., 1982)

Although most hydroxamate siderophores contain three secondary hydroxamate groups, others exist possessing only two. The citrate hydroxamates (Figl.2e) are derivatives of citric acid in which the distal carboxyl groups have been substituted with more effective iron complexing units, i.e. two hydroxamate groups. Aerobactin from *Klebsiella pneumoniae* and plasmid-bearing strains of *Escheria coli*, arthrobactin from Arthrobacter sp. and schizokinen from *Bacillus megaterium* are citrate hydroxamates.

family of coprogens include both dihydroxamate The and trihydroxamate siderophores. The linear ligand molecules of this family contain a diketopiperazine ring formed by condensation of two N^{δ} -hydroxy- N^{δ} acyl-L-ornithine units. In the trihydroxamates, another unit of monomer is ester linked to one end of the dipeptide chain. Rhodotorulic acid (Figl.3a), a dimer of N⁰-hydroxy-N⁰-acetyl-L-ornithine, (Atkin and Neilands, 1968), has been found to be produced by many yeasts and smut fungi, the original producer being Rhodotorula pilimanae. Dimerumic acid (Figl.3b), produced by Fusarium dimerum also rhodotorulic acid moiety. Coprogen, contains the the

predominant siderophore of *Neurospora crassa* is a trihydroxamate siderophore also containing the rhodotorulic acid moiety (Padmanaban and Sarma, 1964). Its structure is shown in Figl.3c.



Fig. 1.2 Structures of some hydroxamate siderophores.a) Ferrichrome b) Albomycin c) Methane sulphonate salt of deferriferrioxamine B d) Fusarinine C e) Citrate hydroxamate siderophores.







Fig. 1.3 Structures of some hydroxamate siderophores belonging to the coprogen family.

a) Rhodoturlic acid b) Dimerumic acid c) Coprogen

Triornicin and isotriornicin, two members of the coprogen family were found to be produced by Epicoccum purpurascens (Frederick *et al.*,1981,1982). The ligand molecules are structurally similar to coprogen, except that the two termini of the molecules have dissimilar N° -acyl groups (one acetyl and trans-anhydromevalonyl one groups). In triornicin, the N^{o} -acetyl group is in the ester-linked ornithine, whereas, in isotriornicin, this group is in the diketopiperazine end.

1.2.2. Phenolates/Catecholates.

The catecholate siderophores are made of three units of 2,3 dihydroxy benzoic acid (DHBA) (occasionally salicylic acid) linked by amino acid and/or amino alkane residues forming either a cyclic or a linear molecular backbone. Each catecholate group provides two oxygen atoms for chelation with iron so that a hexadentate octahedral complex is formed as in the case of the hydroxamate siderophores. Enterobactin, (Figl. 4a), a triester of 2,3- dihydroxybenzoyl serine is an example of a cyclic catecholate siderophore. The ligand was isolated from low-iron cultures of S.typhimurium (Pollack et al., 1970 b) and E.coli (O'Brien and Gibson, 1970) and named enterobactin and enterochelin, respectively. It is the prototype of the catecholate siderophores and forms a complex with iron (III) with the highest formation constant (10^{52}) among all known siderophores (Aveleef et al., 1978; Harris et al., 1979). The molecule may be intracellularly hydrolyzed by Fes-esterase or spontaneously break down in the medium to yield dimers and monomers of dihydroxhybenzoyl serine (DBS), which products are also capable of transporting iron (Hantke, 1990). Klebsiella sp. and E.coli also produce a threonine conjugate analogous to enterochelin while Bacillus Subtilis contains а glycine conjugate.

Agrobactin, isolated from *Agrobacterium tumefaciens* (Ong *et al.*,1979) is a linear tricatecholamide made of three residues of 2,3-DHBA, a threonyl group in the form of oxazoline ring and a spermidine chain (Figl.4b). The x-ray structure of agrobactin has been solved (Eng-Wilmot and van der Helm, 1980).

In parabactin (Fig. 1.4c R=H) isolated from *Paracoccus* denitrificans (Tait, 1975; Peterson and Neilands, 1979), the meta hydroxyl group of the middle catechol ring is absent. Vibrobactin reported from *Vibrio cholerae* (Griffiths *et al.*,1984) differs from agrobactin in having a norspermidine residue instead of the spermidine chain and two oxazoline rings instead of one.

A survey of gram-negative bacterial plant pathogens showed that most strains of A.tumefaciens and A.radiobacter investigated produced catecholate siderophores as did Erwinia carotovora carotovora 78 (Leong and Neilands, 1982). Strains of the freshwater pathogen of fish and humans, Aeromonas hydrophila, also produce catecholate siderophores. The first report of catecholate siderophore production by a strain of A.hydrophila was made by Andrus and Payne in 1982. Enterobactin was identified as the siderophore produced by this strain (Andrus and Payne 1983). Catecholate siderophore synthesis by other strains of this organism could not attributed to be enterobactin and in 1989 the production of a new siderophore, Amonabactin, by Aeromonas hydrophilia 495A2 was described (Barghouthi et al., 1989). Amonabactin is composed of 2,3 DHBA, lysine, glycine and either tryptophan (amonabactin T) or phenylanine (amonabactin P). Amonabactin is produced by many isolates of the genus Aeromonas.

1.2.3. Pseudabactins, Pyoverdines and Mycobactins.

The production of yellow/green fluorescent pigments is a characteristic of some Pseudomonas and Azotobacter species grown under deficient conditions. The compounds form stable red-brown complexes with Fe^{3+} and stimulate iron uptake (Meyer and Hornsperger, 1978 ;Meyer and Abdallah, 1978; Knosp *et al.*,1984). Two different names have been used for the compounds isolated from the pseudomonas species: pseudobactins and pyoverdines. The complete structure of pyoverdine has been determined for a few strains, namely, *P.aeruginosa* ATCC 15692, (Wendenbaum *et al.*,1983) and for soil isolates related to *P.fluorescens* or *P.putida*, namely, strains B10, 7SR1, A214 and

WCS358 (Teintze *et al.*,1981; Yang and Leong, 1984; Buyer *et al.*,1986; van der Hofstad *et al.*,1986). The term pseudobactin has been used for the pyoverdines of these rhizobacteria. These studies show that each of these strains produces a chemically different pyoverdine, with certain features in common. In all cases pyoverdines have a 2.3-diamino-6.7-dihydroxyquinoline derivative, which gives colour and fluorescence to the molecule, linked to a small peptide, which differs among strains in the number and composition of amino acids. A third part of the molecule, usually formed by a succinamide group linked to an amino group of the quinoline derivative, can also differ depending on the strain since it is replaced by a malamide group for pseudobactin A214 (Buyer *et al.*,1986).

Chelation of iron (III) involves the catecholate group of the chromophore and the two hydroxamate moieties of the hydroxyaminoacyl residues of the peptide chain. Thus pyoverdines form a family of compounds which are intermediate between the usually strict catecholate or hydroxamate siderophores.

Pseudobactin from Pseudomonas B10 has the following structure: L-Lys-D-Threo- β -OH-Asp-L-Ala-D-allo-Thr-L-Ala-D-N^{δ}-OH-Orn

(Figl. 4d). The ornithine residue in this hexapeptide is cyclised into an N-hydroxy piperidone ring and the lysine residue is linked to a fluorescent quinoline derivative. from *P.fluorescens* contains Pyoverdine seven amino acid residues with two hydroxamate groups and an 0-dihydroxy It aromatic group. is а linear octapeptide, D-Ser-L-Arg-D-Ser-L-N^b-OH-Orn-L-Thr-L-Thr-L-

lys- N^{δ} -OH-Orn. The two hydroxamic acid groups of the ornithine residues and the O-dihydroxyquinoline molety form the chelating groups for iron.

The mycobactins produced by mycobacteria have the general structure shown in Figl. 4e. Mycobactin is a unique microbial siderophore in that it is both lipid-soluble and intracellular in occurence. It was first isolated as a growth factor for *M.paratuberculosis* and the original work leading to the isolation of a number of related mycobactin molecules was

carried out by Snow in 1970. Mycobactins, containing two hydroxamate groups, the third pair of chelating groups being provided by an oxygen atom on the aromatic residue and a nitrogen on the oxazoline ring, may also be viewed chemically as hybrids between the hydroxamate and phenolate-catecholate classes of siderophores.

In the structure shown $R_1 - R_5$, are substituents which vary according to the species: R_1 is usually an unsaturated alkyl chain $(C_{12}-C_{19})$ and R_2 and R_3 are either -H or $-CH_3$. R_4 is normally $-CH_3$ or $-C_2H_5$ but may be a longer alkyl chain with *M.avium*. R_4 may also be a long alkyl chain $(C_{15}-C_{19})$ with *M.narinum* and with *Nocardia asteroides* (C_9-C_{13}) but in these cases R_1 becomes a $-CH_3$ group. R_5 is either -H or $-CH_3$. Thus, it is now possible to identify most mycobacteria according to the mycobactin which they produce.

As mycobactins occur intracellularly they are thought to act in conjunction with extracellular chelating agents termed exochelins (Stephenson and Rathledge, 1980). The structure of these iron-binding compounds has not been firmly established.

1.2.4. Rhizobactin.

Most fixed nitrogen in the biosphere arises from symbiosis between specific members of the Rhizobia and their respective host plants. Due to the presence of iron at the different stages of the fixation process, the iron metabolism of some species was examined.

Rhizobium meliloti DM4 could be made iron deficient in laboratory culture but tests of the supernatant fluid with the Arnow and Csaky reagents gave negative results for catechol and hydroxamate siderophores respectively. The isolation of rhizobactin became possible once these colourimetric assays were replaced by an equally sensitive but less restrictive EDDA-Luria broth (LB) bioassay (Smith and Neilands, 1984). The compound N²-[2-[(1-carboxyethyl)-amino]ethyl]-N₂-(3-carboxy-3hydroxy-1-oxopropyl) lysine contains ethylene α-hydroxycarboxyl moieties diaminedicarboxyl and as metal-coordinating groups. The ethylenediamine group is novel

as a natural product and is unprecedented as a ligand in the siderophore series, which characteristically contain catechol or hydroxamate functional groups. This genus and the siderophores discovered to date will be discussed at a later stage. The structure of Rhizobactin is given in Figl.4f.



Fig. 1.4 Structures of siderophores belonging to the catecholate and rhizobactin class of siderophores.a) Enterobactin b) Agrobactin c) Parabactin d) Pseudobactin

e) Mycobactin f) Rhizobactin

1.3 Microbial proteins involved in iron acquisition.

As stated previously, the efficient absorption at low concentrations of the various siderophores produced by microbes under iron stress is achieved by receptors in the cell envelope. The requirement for a receptor follows logically from the observation that water-soluble compounds exceeding ca. 500 to 600 daltons cannot permeate the small water filled pores in the sealed outer membrane of gram-negative bacteria.

Most of the information about these receptor proteins has been collected through studies on bacteria, especially enteric species such as *E. coli and Salmonella typhimurium*. This is due mostly to the more complete understanding of the genetics of the enterics as a class, as contrasted to other bacterial species or fungi.

Genetic and biochemical analyses of the cell envelopes of gram-negative bacteria, in particular those of E.coli and S.typhimurium, have revealed subs intial detail regarding the morphology, composition and func ion of the triple-layered shell structure of these organisms Inouye, 1979). The inner or cytoplasmic membrane is a phospho ipid bilayer containing a large number of peripheral and int gral proteins. The major functions of these are the active transport of substrates, including iron, and energy transduction (Rosen, 1978). In the periplasmic space between the inner and outer membranes lies a peptidoglycan or murein network that imparts shape and rigidity to the cell (Henning, 1975). In this peroplasmic space are found proteins involved in binding or processing of transported solutes (Dills et al., 1980). The outer membrane, in which most the iron-related proteins have been detected, is an of asymmetric bilayer with chains of lipopolysaccharide protruding from the outer leaf. The molecular weights of the iron-related proteins, in the range of ca. 80,000 daltons by SDS-PAGE analysis, are such that they can easily span the periplasmic space and contact the inner membrane.

The actual identification of a siderophore receptor, first achieved for ferrichrome (Wayne and Neilands, 1975), owes much to work on vitamin B12 transport in *E.coli* and the

demonstration that the vitamin and colicin E share a common binding site. Also essential was the discovery of a series of sid mutants of S.typhimurium resistant to an antibiotic analogue of ferrichrome, albomycin (Luckey et al., 1972). The mutants were incapable of ferrichrome transport and were mapped at the pan locus on the chromosome. The pan locus in E.coli coincides with the classic genetic lesion, ton A, and this was instrumental in generating the thought that the biochemical function of its gene product must be that of a receptor for ferrichrome (Wayne and Neilands, 1975). Therefore E.coli, even though it does not synthesize ferrichrome, maintains a recptor for its uptake. Once the ton A (now fhu A) protein had been identified as a component of the ferrichrome receptor, it was assumed that receptors for other siderophores should exist and that these in many instances might also serve as binding sites for specific phages and bacteriocins. Thus, the protective effect of ferric enterobactin against colicin B, first shown by Guterman (1973), could be ascribed to competition for a common binding site in the outer membrane.

E.coli does not synthesize or normally require B12, although it maintains both an uptake system for the vitamin and at least two enzyme proteins that use it as a cofactor. The genetic organization of the vitamin B12 transport system is shown in Figl.5.

Gene *btuB* codes for a 66,000-dalton outer membrane receptor that requires a divalent cation and lipopolysaccharide for maximum activity. As well as B12 it is also a receptor for colicins E and A and phage BF23 (Heller and Kadner, 1985) although modification of the *btuB* cistron via the *btuA* mutation impairs B12 transport without affecting sensitivity to the E colicins or phage BF23. This leads to the conclusion that different binding sites occur in the receptor. Available evidence suggests that the products of the *btuCED* operon comprise a periplasmic binding protein-associated system for transport of vitamin B12 across the cytoplasmic membrane (Reynolds et al., 1980; DeVeaux et al., 1986). Finally, intact ton B and exbB genes are required for B12 uptake and sensitivity to many phages and colicins. Iron chelate uptake systems also depend on the function of the tonB gene product (Frost and Rosenberg, 1975; Bassford et al., 1976). Studies on mutations in this gene led to the proposal that the tonB product couples metabolic energy to the outer membrane receptors responsible for the binding and uptake of these nutrients, colicins, and phages (Reynolds et al., 1980). The B12 system of uptake remains the model that most closely parallels siderophore transport, including the participation of the tonB gene function (Neilands, 1982).

The essential features of the high affinity iron assimilation system are depicted in Figl. 6.

At low levels of available iron in the growth medium, which generally means less than about micromolar, the biosynthetic machinery for both siderophore and receptor is derepressed. The function of the receptors is to bring the ferrisiderophore to, or through, the envelope where the metal ion undergoes a reductive separation from the ligand.

1.4 Iron transport systems in E.coli

The enteric bacterium *E.coli* K-12 is the single microorganism for which the most information as regards its genetic constitution is known. It is also the bacterium that is the focus of studies designed to elucidate the molecular mechanism of iron assimilation and the regulation of this process.

When one considers that *E.coli* K-12 can synthesize and transport ferric enterobactin, can transport exogenous siderophores such as ferrichrome and ferric rhodotorulate, can induce a system for transport of ferric citrate, may harbour plasmids for synthesis and transport of ferric aerobactin and additionally may assimilate iron via a relatively inefficient low-affinity pathway, one is presented with an extremely complex system of iron assimilation - iron is obviously not to be excluded from the dietary regime of *E.coli*.

1.4.1. Ferrienterobactin transport in E.coli.

The endogenous high affinity transport system for iron of *E.coli* is mediated by the siderophore enterobactin, a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine (DBS) that binds one atom of ferric iron. A view of the ferrienterobactin transport system is shown in Fig. 1.7.



Fig. 1.6 Essential features of the high affinity iron assimilation system

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Enterobactin is synthesized and released into the medium when E.coli grows in an iron-deficient medium. Ferric-enterobactin complexes form and these are transported through the cell envelope into the cytoplasm. Iron is made available to the cell and the enterobactin is hydrolysed. The products of at least seven biosynthetic genes and five transport genes are required for this process. The genes, all of which map at around 13 min on the E.coli chromosome, occur in the following order; entD fepA fes entF fepE fepC fepD fepB fepF entEBG (AC) (Fleming et al., 1985; Nahlik et al., 1987; Pierce and Earhart, 1986). Genes disignated ent are involved in enterobactin (Ent) biosynthesis, fep genes are involved with FeEnt transport, and the fes gene specifies the ferrienterobactin esterase which is necessary to make siderophore-bound iron available to the cell. The pathway for Ent synthesis and degradation is shown in Fig. 1.8.


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Fig. 1.8 Pathway for enterobactin synthesis and degradation

The pathway starts with chorismic acid, the branch point in aromatic acid biosynthesis. The products of entC, B and A then convert chorismate to DHB (Young et al., 1969 a,b, 1971). The synthesis of Ent from DHB and L-serine is accomplished by the products of genes entD, E, F and G (Woodrow et al., 1979). The EntD, E, F and G polypeptide are believed to associate to form enzyme complexes (Greenwood and Luke, 1976 and 1980) and it has been suggested that the Ent synthetase complex is associated with the cytoplasmic membrane, which might offer the means to excrete Ent thus avoiding degradation by Fes and possible intracellular damage due to iron chelation by uncomplexed Ent. Except that FepA (the product of the fepA gene) is not required, there is little known about how Ent leaves cells. The products of the fepA, fepB and fepC genes are required for transport of FeEnt through the cell envelope as are the products of the tonB and exbB genes which map at 28 min and 59 min, respectively.

FepA: FepA, an 81,000 Mr outer membrane protein, serves as the receptor for FeEnt and also for colicins B and D. Both functions are separable by mutation (McIntosh *et al.*, 1979), as is the case for the vitamin B12 receptor. The synthesis of FepA is coordinately controlled in *E.coli* K-12 with that of two other outer membrane proteins, Cir (Mr 74,000) and Fiu (Mr 83,000). The FepA, Fiu and Cir proteins are dominant members of the outer membrane polypeptide complement under conditions of iron starvation. The *cir* gene maps at 43 min and its product functions as the receptor for colicins la and lb (Cardelli and Konisky, 1974). No function is known for the *fiu* gene product (18 min) (Hantke, 1983). Why these proteins are coordinately regulated is not known.

FepB: In 1983 genetic proof was provided for the existence of at least two *fep* genes (Pierce *et al.*,1983). The strain DK214 overproduces Ent and is defective in FeEnt transport and fails to grow in iron-deficient media. The lesion in DK214 was complemented by hybrid λ phage Al but this failed to complement strains bearing *fepA* mutations. The new gene, named *fepB* was shown to map between *entF* and *entA*. The *E.coli* DNA fragment in λ Al was cloned into the vector pACYC184 giving plasmid pCP111 (Pierce and Earhart, 1986). FepB was identified using minicell, maxicell and in vitro DNA-directed protein synthesizing systems. The *fepB* gene product gives rise to four polypeptides, Mw. ranging from 31,500 to 36,500 on SDS-polyacrylamide gels. Localization studies determined that the largest *fepB* product, proFepB, is membrane associated whereas the other forms of FepB are found in the periplasm. The evidence indicates that *fepB* codes for a periplasmic protein and that proFepB posesses a leader sequence. That FeEnt transport requires a periplasmic protein emphasizes the similarities between vitamin B12 and FeEnt transport.

FepC: Studies on subclones of pCP111 revealed the presence of another gene (fepC) required for FeEnt transport (Pierce and Earhart, 1986). FepC has an Mr of 30,500 on standard SDS-poylacyrlamide gels. No proFepC product was observed in mini or maxi cells and FepC is a membrane constituent probably located in the cytoplasmic membrane since such proteins are synthesized without a leader sequence. There is at least one other polypeptide encoded by plasmid pCP111. This protein FepF is a membrane component with a Mr of 29,500. The gene is between fepB and entE and this suggests that it may have a role in FeEnt transport since thus far only ent-related genes have been detected in this region.

Mutations in tonB were isolated over 40 years ago as a TonB: class of E.coli surviving exposure to bacteriophage T1 (Luria and Delbruck, 1943). They have been subsequently shown to be to bacteriophage ϕ 80, (Matsushiro, 1963), insensitive insensitive to group B colicins, defective in vitamin B12 transport (Bassford et al., 1976) and defective in all high-affinity siderophore mediated iron transport. The cytoplasmic membrane can generate and maintain an electrochemical potential sufficient to energize the active

transport of nutrients across it. Because small molecules freely diffuse across the outer membrane it cannot generate or maintain an electrochemical potential sufficient to energize the processes mentioned above. TonB is proposed to function in these processes as an energy transducer, coupling cytoplasmic membrane energy to energy-dependent outer membrane phenomena (Hancock and Braun, 1976). Definitive evidence concerning the location of TonB is lacking. A cytoplasmic location has been reported (Plastow and Holland, 1979) but a periplasmic location has been argued due to the presence of a leader sequence and evidence that TonB is hydrophilic (Postle and Good, 1983). The TonB product is unstable and has a short functional half-life in the above processes (Kadner and McElhaney, 1978). This has been interpreted as evidence that a limited amount of TonB exists and that TonB is consumed while carrying out its function.

ExbB: Like the *tonB* gene, *exbB* is not located in the Ent gene cluster. There is a dispute regarding the role of ExbB in ferrichrome uptake (Hantke and Zimmerman, 1981; Pugsley and Reeves, 1976a) but it is agreed that cells with *exbB* mutations are partially defective in FeEnt uptake, hyper excrete Ent, exhibit strongly decreased Bl2 transport and are sensitive to group B colicins (Guterman and Dann, 1973; Pugsley and Reeves, 1976 b). The role of ExbB is unknown.

1.4.2 Regulation of the Enterobactin System

Regulation of FeEnt transport is under the control of the fur (ferric uptake regulation) gene, as are all high affinity transport systems for iron. Mutations in fur were first identified in S.typhimurium (Ernst et al., 1978). These cells constitutively expressed with fur mutations three iron-regulated outer membrane proteins, Ent synthesis, FeEnt and ferrichrome uptake and degradation of intracellular FeEnt. Using the operon fusion fhuA-lacZ (fhuA = tonA: for ferric hydroxamate uptake), similar mutants were isolated in E.coli (Hantke, 1981). In this case, fur was shown to enhance fepA transcription by a factor of three under iron replete

conditions. A fur:: Tn5 mutation was isolated in 1985 (Bagg and Neilands, 1985) using a plasmid containing a fusion of lacZ and a gene for aerobactin synthesis. Using this *iucC-lacZ* (iron uptake chelate) fusion, mutants derepressed for B-galactosidase synthesis under iron-replete conditions were isolated. The fur:: Tn5 mutant was found to be derepressed not only for the synthesis of B-galactosidase, but also for the synthesis of catechol and hydroxamate. Outer membrane proteins normally derepressed under low iron condition were also constitutively derepressed giving a phenotype characteristic of previously described fur mutants. All fur mutations were placed at about 15.7 min on the E.coli chromosome. Fur appears to direct the synthesis of an 18,500 Mr polypeptide (Hantke, 1984) and the Fur protein is thought to act as an aporepressor, with iron or an iron-related compound acting as co-repressor. Expression of the Ent biosynthetic genes is also strongly regulated by iron. for coordinated The mechanism the regulation of the biosynthetic genes is not known but the product of the fur gene also plays a role.

1.4.3. Regulation of the tonB gene.

Under aerobic conditions, regardless of changes in iron availability or the fur locus, the tonB gene was thought to be transcribed constitutively (Dorman et al., 1988) even though sequences similar to other Fur-binding sites (deLorenzo et al., 1987; Griggs and Konisky, 1989; Pressler et al, 1988) are present in the tonB promoter region (Postle and Good, 1983). In a study by Postle (1990) the issue of *tonB* transcriptional regulation was reexamined using three different tonB-lacZ transcriptional fusions differing in the extent of intervening lacZ. with DNA between tonB and The results the under tonB'-Mu-trpC'-trpB-trpA'-lacZ fusion show that iron, β -galactosidase remained conditions of excess at approximately 300 U throughout the growth of the culture whereas under conditions of iron starvation, β -galactosidase levels were approximately twofold greater at all points compared with iron excess conditions. β -galactosidase levels in a fur::Tn5 derivative of this strain were elevated approximately twofold in the presence of 100 μ M FeCl₃ compared with the fur⁺ derivative, suggesting that the iron-dependent regulation observed requires an intact fur locus. This is the first report of regulation of TonB by iron..

1.4.4. Aerobactin iron uptake system.

In 1979, Williams reported the presence of a hydroxamate type of siderophore in certain clinical isolates of *E.coli* harbouring the plasmid coding for colicin V. The isolates were characterized by their capacity to cause disseminating infections in experimental animals. It was shown that the virulence attribute could be assigned to the siderophore rather than to the bacteriocin colicin V or to enterobactin, the siderophore indigenous to practically all enteric bacteria.

A general relationship between the sensitivity to bacteriocins or phage and the presence of envelope components conferring the ability to transport specific ferric siderophores was proposed in 1975 by Wayne and Neilands, following the demonstration that the ability of *E.coli* to transport ferrichrome and its sensitivity to a group of lethal agents including colicin M, albomycin and bacteriophages T1, T5 and ϕ 80 was linked to a single gene, namely, tonA (fhuA). A similar correlation between aerobactin tranpsort and sensitivity to cloacin, a bacteriocin produced by Enterobacter cloacae was established, (Van Tiel-Menkveld, 1982), thus paving the way for cloning the aerobactin operon by screening for acquired sensitivity to (Bindereif Neilands, cloacin and 1983). А plasmid, pColV-K30, approximately 100kb in length was isolated and digested with Hind III. The resulting fragments were ligated into the Hind III site of the multicopy vector pGL102. Two recombinant plasmids, pABN1 and pABN5 were obtained and found to harbour, respectively, the complete biosynthesis plus transport genes(pABN1) and only the biosynthetic genes for aerobactin. The plasmid, pABN5, a subclone of pABN1 was generated by splitting the larger insert with EcoRl. It seemed likely therefore that the EcoRl site occured in the receptor

gene and that the entire aerobactin biosynthesis and tranpsort complex was packaged on not more than 7 - 8 kb of DNA. Both plasmids also contained the main iron-regulated element.

Several years of experimentation had to be carried out in order to sort out the various biosynthetic genes and to assign a function to each. It was established that the biosynthesis of aerobactin should require a minimum of three steps starting from lysine and citrate (Fig. 1.9) and involve not less than this number of proteins.





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Preliminary analysis by SDS-PAGE of polypeptides generated in minicell preparations revealed the presence of 74kDa, 63kDa, 53kDa and 33kDa bands (Bindereif et al., 1983). The genes were designated in linear order as, iucA, iucB, iucD, and iutA, iuc and iut referring to iron uptake chelate and iron uptake transport, respectively. The 74kDa protein was the outer membrane receptor and enzyme assays identified the 33kDa protein as the acetylase (de Lorenzo et al., 1986). The 53kDa band was assigned the function of the oxygenase (Gross et al., 1985). The 63kDa band was identified as the α subunit of the synthetase, while a 62kDa band, (the product of the iucC gene), long obscured by the strongly expressed 63kDa band, was idenitified as the β subunit (de Lorenzo and Neilands, 1986). Fig.(1.10) shows the organization of the pColV-K30 aerobactin gene system.



63K 33K 62K 53K 74K pColV-K30

63K = Synthetase (subunit α) 33K = Acetylase 62K = Synthetase (subunit β) 53K = Oxygenase

74K = Receptor

Examination of the sequences flanking the iron uptake region of pColV-K30 revealed the presence of repeated sequences homologous to ISI and, in addition, two replication regions were also found at both ends of the aerobactin iron uptake region. These studies were carried out in an effort to understand the location of the aerobactin system, not only on ColV-type plasmids but also its location, in the majority of *E.coli* K1 strains associated with human neonatal infections, on

the bacterial chromosome (Valvano and Crosa, 1984; Valvano et al., 1986). Some species of Shigella such as S.flexneri and S.boydii also possess a chromosome mediated aerobactin system (Lawlor and Payne, 1984). Plasmids other than ColV-K30 were also reported to possess aerobactin sequences, i.e., pRJ100 (Stuart et al., 1980) and ColV-K311 (Braun et al., 1983) in E.coli, pSMN1 in Enterobacter aerogenes and pSMN2 and pSMN3 in Salmonella arizona (McDougall and Neilands, 1984). The aerobactin regions appear to be highly conserved in these genomes, promting the suggestion that the aerobactin genes may be highly mobile as a recombinational unit, and they may have integrated at different sites in various genomes (Perez-casal and Crosa, 1984). In 1979, inverted IS1 sequences were reported to flank the gene for the heat-stable enterotoxin, an important virulence factor that has spread to various medically important microorganisms (So et al., 1979). In this case the whole unit, heat-stable enterotoxin genes together with the flanking IS1 elements, was shown to have transposition ability. Although this has not been demonstrated for the aerobactin region, there is potential for such an activity, since the IS elements flanking the region are themselves capable of transposition. Another possibility is that the presence of IS1 elements could enhance the ability of the aerobactin region to recombine by homologous recombination via the IS elements, with genomes that also possess IS1 sequences. This hypothesis is strengthened by the finding that in an E.coli Kl strain isolated from a case of human neonatal meningitis, the aerobactin regions are located on a 10.5kb chromosomal Hind III fragment that also carries IS1-like elements (Valvano and Crosa, 1984). The REP1 and REP11 replication regions that flank the aerobactin system sequences may contribute to their conservation since recombinational events that conserve one of these replication regions may also leave intact the aerobactin system, especially in deletion or insertion events in which new plasmids are generated. The combination of replication regions and insertion sequences adjacent to the aerobactin sequences may have played a role in their preservation during evolution and may have contributed to

their spread.

1.4.5. Regulation of the aerobactin operon of pColV-K30.

The promoter region of the pColV-K30 encoded operon was located by S1 nuclease mapping and shown to have two start sites, a strong one detected both in vivo and in vitro and labelled Pl and a weaker one, designated P2, only detected in vitro and located about 50bp further upstream (Bindereif and Neilands, 1985). To confirm that this promoter region was the site of regulation by iron, a protein fusion was prepared in which lacZ, minus its promoter, was ligated to iucA. The resulting protein fusion was found to induce the expression of β -galactosidase to a level of about 1,200 units per absorbancy units at 600nm when the cells were depressed by additon of excess bipyridyl to scavenge iron. The relative strength of the aerobactin promoter enabled application of a novel quantitative S1 nuclease protection assay to prove that regulation occurs at the transcriptional level. The promoter was localized to a 0.7kb Hind III - Sall fragment by in vitro transcription. Total RNA was isolated from *E.coli* BN3040 Nal^r(pColV-K30), which carries the aerobactin gene complex in a single copy per cell, after growth in iron replete, iron limited and iron starved conditions. The total RNA was used in an S1 nuclease assay with the single-stranded 0.7kb Hind III - Sall probe 5' end-labeled at the Sall site. The products of the Sl nuclease digest were analysed on denaturing gels. Under iron replete conditions. no specific RNA initiated at the major promoter could be detected and under iron-limiting conditions a very low level of specific RNA existed while under iron starved conditions the level of transcripts increased by more than 10-fold, proving directly that the levels of RNA initiated at the major promoter are regulated by iron.

In 1987, de Lorenzo *et al.*, carried out work to define the smallest region of the aerobactin promoter capable of iron regulation of an *iucA'-'lacZ* gene fusion and to detect the actual contact of the operator sequences with the Fur protein in the presence of a number of divalent heavy-metal cations.

Analysis of a collection of gene fusions linked to Bal31 deletions through the promoter region limited the putative regulatory sequences to a 78bp insert of pColV-K30 in pDB37. In the presence of a number of divalent heavy metals (Mn^{2+}, Fe^{2+}) , Co^{2+} , Cu^{2+} , Cd^{2+} and partially with Zn^{2+}), Fur binds primarily to a DNA sequence of 31bp within the promoter region. The absence of divalent metals results in a dramatic decrease in the DNA-binding ability of the repressor. Although all the metals assayed activated the repressor, the effects were not equivalent. The results with Mn. Fe and Co were clear-cut and indicated that any of these could repress the expression of the β -galactosidase gene placed under control of the iron-sensing aerobactin promoter. Other metals gave ambiguous results. The results with iron were only observed when oxygen was excluded thus indicating that iron is the biometal involved in the regulation. DNasel footprints revealed that the upstream boundary of the protected region is just 2bp downstream from the start of the insert sequence in the smallest plasmid deletion (pDB37) which showed iron control of the corresponding gene fusion. The experiments described demonstrate that Fur is а DNA-binding protein and that it requires a divalent heavy-metal ion as an activator. Fur is a negative regulator of expression and it is a repressor which binds Fe²⁺ as corepressor.

1.4.6 Independence of the aerobactin and enterobactin iron uptake systems in *E.coli*.

As mentioned previously, many strains of E.coli isolated from extraintestinal infections of humans and domestic animals are synthesize siderophores, aerobactin able to two and enterobactin. Although aerobactin has a dramatically lower (10^{23}) 10^{52} enterobactin and affinity for iron than respectively), it has been shown to provide a significant selective advantage for bacterial growth in conditions of iron limitation that exist in the body fluids and tissues of an infected animal due to the presence of the iron binding lactoferrin glycoproteins transferrin in serum and in secretions.

Using as a probe, the antitumour agent, streptonigrin, which is bactericidal in the presence of iron, Williams and Carbonetti (1986) determined levels of intracellular iron during bacterial growth promoted by the two siderophores. A strain producing only enterobactin remained sensitive to streptonigrin, suggesting that assimilated iron was contributed to an intracellular pool, while a strain synthesizing only aerobactin resistant to streptonigrin, indicating that became iron complexed with aerobactin may be chanelled directly to where it thus making is required for growth it unavailable streptonigrin. These results suggest that with aerobactin there is a more efficient utilization of the iron assimilated. Another reason put forward regarding the selective advantage conferred on strains producing aerobactin is that aerobactin is more effective than enterobactin at very low concentrations of siderophore. This is likely to be an advantage in the fluid environments of the bloodstream and the urinary tract in which any extracellular bacterial product will be continually diluted. This difference between the two siderophores may be due to the fact that while enterobactin is enzymatically degraded during intracellular release of ferric ions and therefore wasted (Rosenberg and Young, 1974), iron is more readily released from aerobactin and the siderophore may subsequently be resecreted (Braun et al., 1984). Williams and Carbonetti (1986) also suggest that the relative streptonigrin resistance of a strain that is able to synthesize both aerobactin and enterobactin growing in the presence of transferrin and lactoferrin at only partial iron saturation, preferentially active aerobactin is indicates that in conditions of iron stress. It was found that only in conditions of extreme stress imposed by the presence of α , α' -dipyridyl at a concentration of 50μ M or greater was significant activity of the enterobactin system apparent. This may reflect differences in genetic and regulatory complexity. It has been suggested that the genetic determinants of aerobactin biosynthesis and uptake are expressed more readily than those of enterobactin in

response to iron limitation. In 1979 work carried out by Williams showed that in a mutant strain of *E.coli* K-12, AN1937, deficient in enterobactin biosynthesis but carrying the ColV plasmid, the outer membrane proteins characteristically induced in condition of iron stress are repressed. This implies that synthesis of adequate levels of aerobactin to maintain maximal growth is possible even when other iron-regulated gene expression is repressed.

In a study carried out in 1982 by Stuart et al., where 476 strains of *E.coli* isolated from humans, pigs, cattle, poultry, potable water, or effluent were examined for low-iron induced ability to produce hydroxamate, (Hyd), it was concluded that the ability to carry out hydroxamate-mediated transport of iron is widely distributed among natural isolates of E.coli but that the distribution is not random. E.coli isolated from sources where levels of available iron might be expected to be low tend to be Hyd⁺. One such source is the faeces of breast fed babies. E.coli isolated from this source tended to be Hyd^{\dagger} and this is consistent with the view that iron present in breast milk is not readily available. In contrast, the low proportion of Hyd^{\dagger} strains among the isolates from potable water and effluent suggests that monitoring of the Hyd^{\dagger} phenotype can provide a useful indicator of the availability of iron for microbial growth in a particular situation. Finally, in this study, a significant difference was observed between the proportion of Hyd^{\dagger} among Col⁺ and Hyd⁺ among Col⁻ E.coli isolated from human bacteremia. Aerobactin, therefore, is established as а virulence determinant and as an effective chelator of iron surpassing the enterobactin-dependent system with regard to supplying sufficient iron to E.coli cells growing in animal fluids.

1.5. Siderophores and virulence.

Bacterial pathogenicity is the result of many parameters working together to establish the pathogen in the host vertebrate such as, bacterial attachment, inhibition of phagocytosis, complement-dependent bacterial killing and toxin production, to mention a few (Ellwell and Shipley, 1980). However virulence can only be possible once an organism has established growth, especially in invasive type infections such as those resulting in sepsis, where the invading microorganism must reach relatively high concentrations in body fluids and tissues. Bacterial growth depends on the ability to acquire iron which is not readily available in the host vertebrate since it is complexed to high affinity iron-binding proteins. The possession of specialized iron transport systems may be crucial for bacteria to override the iron limitation imposed by the host.

The plasmid encoded pColV-K30 mediated iron transport system of E.coli has already been discussed. In the fish pathogen, Vibrio anguillarum, the iron transport system is also plasmid encoded. Vibrio anguillarum causes vibriosis, a very serious infectious disease affecting marine fish characterized as a haemorrhagic septicemia. It was determined that the high virulence phenotype of many strains of V. anguillarum could be ascribed to the presence of a 65kb plasmid designated pJM1, the representative plasmid having been isolated from V. anguillarum 775 (Crosa et al., 1977, 1980). This plasmid was shown to code for a very efficient iron-uptake system that permits bacteria to grow under iron-restricted conditions (Crosa, 1980). The iron uptake system was analysed using mutations caused by the transposon Inl (Walter and Crosa, 1983). Two plasmid-mediated components involved in the process of iron uptake in V. anguillarum were defined, a siderophore and its cognate receptor for the uptake of the iron siderophore complex. Tnl induced mutants exhibiting phenotype siderophore-negative receptor-positive were а identified as possessing insertions in a 19.9kb BamHI fragment. A deletion affecting portions of this fragment resulted in a siderophore-negative receptor-negative phenotype. Until 1985 it was not known whether the receptor OM2 was actually encoded by the pJMl plasmid or if it was a chromosomal product regulated by a plasmid specific substance. Work was carried out by Actis et al., in 1985, whereby pJM1 DNA fragments were cloned into cosmid vectors and transferred to E.coli. They showed that the

OM2 protein was synthesized in *E.coli*, demonstrating that it was in fact encoded by pJM1. OM2 has a size of 86 kilodaltons and it is inducible under conditions of iron limitation. The recombinant plasmids were mobilized to a plasmidless strain of *V. anguillarum*, H775-3, via the plasmid pRK2013. It was found that receptor activity and iron-regulated OM2 synthesis is recovered upon the introduction of pJHC-T7 (Fig.1.11). This is not the case with pJHC-A100 which codes for a protein that is immunologically and electrophoretically indistinguishable from OM2 but is no longer under iron regulation. One possible explanation is that, in addition to the OM2 protein, another product encoded in the PJM1 DNA included in pJHC-T7 but absent from pJHC-A100 is necessary for iron transport. The requirement of at least two genes for functional iron transport is not unusual and has been described for other iron uptake systems.



Trans acting factor: Vibrio anguillarum strains harbouring recombinant clones containing the pJM1 iron uptake region produce normal levels of siderophore activity and are iron uptake proficient only if they carry, in addition, another pJM1 plasmid derivative such as pJHC-9-8, in which the iron uptake region has been deleted (Tolmasky and Crosa, 1984). It was apparent, therefore, that a pJM1 region other than the iron uptake sequences encoded a trans-acting factor required for full expression of the pJM1 system. Work carried out by Tolmaksy *et al.*(1988), gave further evidence for the existence of a trans-acting factor. By cloning specific regions of pJM1, recombinant plasmids were obtained carrying the genetic determinants for the Taf (trans acting factor). Quantitation of anguibactin (the siderophore of V. anguillarum) production by bioassays and analysis by spectrophotometric procedures supported the idea that the Taf functions as a trans activator for siderophore biosynthesis. Culture supernatants from strains carrying the iron uptake clone pJHC-T7 either alone or with pJHC-T6.14 which carries the cloned Taf genes, were subjected to the procedure for anguibactin purification. In bioassays, it was shown that the levels of anguibactin activity were 20-fold lower when the Taf genetic determinants were not present. It is likely that regulation of anguibactin gene expression by the trans activator is at the transcriptional level but further characterization is needed to determine the molecular nature and precise site of action. This is the first report of a trans activator for procaryotic iron uptake via stimulation of the siderophore biosynthetic genes.

That the siderophore, anguibactin, which has been shown to be a catechol, (Actis et al., 1986), is produced in vivo was demonstrated by complementation experiments (Wolf and Crosa, 1986). Complementation was obtained in vivo of an iron uptake deficient mutant of V. anguillarum by establishing experimental infections with mixtures of this mutant and a wild type V. anguillarum strain that is iron uptake proficient and produces siderophore. The results of the mixed infections indicated that both the wild type strain as well as the mutant could be recovered from moribund fish. Results obtained with mixtures of the wild type strain with a mutant deficient in both siderophore and receptor activity indicated that only the wild type bacteria could be recovered. Thus it appears that the siderophore anguibactin is produced in vivo and secreted to the fish fluids to scavenge the iron from iron-binding proteins. In a situation similar to E.coli it has been demonstrated that

certain virulent strains of V.anguillarum that do not possess a pJM1-like plasmid are iron proficient and possess chromosomal DNA sequences homologous to pJM1 plasmid DNA sequences (Toranzo *et al.*,1983). This mobility of plasmid DNA is similar to that found with the pColV-K30 region already discussed.

A new iron uptake system was recently described in certain pathogenic strains of *V.anguillarum* (Lemos *et al.*, 1988).This system is chromosomally encoded and differs from the plasmid encoded anguibactin-mediated system in several properties. Anguibactin is not utilised as an external siderophore and although characteristic outer membrane proteins are synthesised under iron deficient conditions, they are not related to OM2. The siderophore produced by these plasmidless strains may be functionally related to enterobactin, as demonstrated in bioassays with enterobactin deficient mutants, although its behaviour in chemical treatments suggest that it is different. Genetic and biochemical characterisation of this system has to be completed in order to assess the relationship to both the enterobactin- and the anguibactin-mediated systems as well as its role in pathogenicity.

Examination of other Vibrio species has shown that a variety of siderophores are produced by these bacteria, although only one of these compounds, vibriobactin, has been purified and its structure determined (Griffiths et al., 1984). In Vibrio cholerae, the synthesis and transport of vibriobactin does not appear to be essential for virulence (Sigel et al., 1984). V.cholerae is a surface pathogen which causes disease by binding to the brush border of the small intestine and producing a potent enterotoxin. It is possible that V.cholerae is able to obtain sufficient iron in the small intestine and does not require its high affinity system. The iron transport system may be more important for the surival of vibrios between hosts. The role of siderophores in virulence of other vibrio species has not been determined, although those vibrios which cause more invasive diseases than cholera are likely to require either siderophores or other specific systems for using host iron sources.

The role of iron transport systems is less clear when the pathogens are non-invasive or predominantly intracellular. Studies on *Salmonella typhimurium*, which produces the phenolate siderophore enterobactin, have shown that the siderophore is not essential for virulence of this intracellular pathogen in mice (Benjamin *et al.*, 1985). Similarly, neither Yersinia pestis nor Legionella pneumophila appear to produce siderophores, but both are pathogenic (Perry and Brubaker, 1979; Reeves *et al.*, 1983). This suggests that mechanisms other than siderophores may be associated with iron acquisition in the intracellular environment. In the case of Y.pestis, it is likely that the source of iron in the host cell is haem, since virulent Y.pestis cells bind and transport haem (Perry and Brubaker, 1979).

In Shigella species, the causative agents of dysentery, a number of differences have been found in the types of siderophores they produce. Enterobactin is synthesised by Shigella dysenteriae and Shigella sonnei but is only rarely detected in Shigella flexneri and Shigella boydii isolates, typically utilize which the hydroxamate aerobactin. The aerobactin genes are chromosomal in the Shigella species unlike the plasmid encoded genes of E.coli ColV. A comparison of the plasmid genes and the genes cloned from the three Shigella species that possess them. show remarkable similarities although it appears that aerobactin is not an essential virulence factor in Shigella. The cloned Shigella aerobactin genes were mutated by the insertion of Tn5 in an aerobactin biosynthesis gene. Marker exchange allowed the insertion of mutated genes into a wild type background (Payne, 1987). The mutants were compared to the wild type for chick embryo lethality and production of keratoconjunctivitis in guinea pigs both of which are used to determine invasiveness of Shigella strains. Although the mutants grow poorly in low iron environments in vitro, no significant differences were seen in the ability of an aerobactin biosynthesis mutant to invade or produce disease in experimental infections. It is possible that siderophore synthesis is important during colonization or establishment of infection but is not required for growth of the bacilli within the epithelial cells. The Shigella species, like, Y. pestis, may utilize haem as an iron source within cells since Shigella binds haemin and can use it as an iron source in vitro (Lawlor et al., 1987).

While it seems that a possible role for siderophores in mucosal and intracellular infections is uncertain, it must be noted that some organisms that cause infections of host fluids produce no detectable siderophore. *Neisseria meningitidis* obtains iron directly from transferrin, lactoferrin, haem and haemoglobin, as does *Haemophilus influenzae* (Herrington and Sparling, 1985). *Streptococcus mutans* produces no apparent siderophore, the organism having only a ferrous transport system and a mechanism for external reduction of iron (Evans *et al.*, 1986). *Listeria monocytogenes* excretes a reductant that removes iron from transferrin.

While there is considerable evidence that competition for iron is a crucial part of the establishment of bacterial infections and that in the case of *Vibrio anguillarum* and certain invasive strains of *E.coli* the production of siderophore is the major virulence determinant, it is likely that many organisms have developed alternative iron sequestering systems and pathogenic iron acquisition must be worked out on a case-by-case basis.

1.6 Rhizobacteria and Siderophores.

The work presented in this thesis is concerned with the production of siderophores by the genus *Rhizobium*, a member of the family *Rhizobiaceae*. While comparitively little is known about siderophore production in *Rhizobium* species, another agronomically important group of rhizobacteria, the pseudomonads, has been the focus of much interest over the last ten years.

1.6.1 Siderophore production by *Pseudomonas* in the rhizosphere.

One of the major taxonomic criteria for the recognition of fluorescent *Pseudomonas* strains is the production of a characteristic yellow-green, water soluble fluorescent pigment named pyoverdine (Elliot, 1958). For a few strains belonging to different species, i.e., *Pseudonomas fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas syringae* and *Pseudomonas putida*, it has been demonstrated that pyoverdine is a powerful iron (III)

chelator, which enables the bacteria to fulfill their iron requirement when growing in an iron deficient environment (Cody and Gross, 1987; Cox and Adams, 1985; Torres *et al.*, 1986; Meyer and Abdallah, 1978). The biosynthesis of this compound is strongly regulated in an inverse relationship by the iron concentration of the bacterial growth medium (Lenhoff, 1963) and pyoverdine has been shown to have a stimulating effect on cell iron uptake (Meyer and Hornsperger, 1978).

The introduction of certain fluorescent *Pseudomonas* spp. into the rhizosphere in short rotation soil has been shown to lead to remarkable increases in crop yield in pot and field experiments (Schroth and Hancock, 1982; Kloepper *et al.*, 1980). Short rotation, which is the frequent cropping of a vegetation in monoculture in the same soil, results in severe losses in crop yield. Continuous growth of potato results in yield decreases up to 30.In most of the Netherlands, for example, growth of potato even once every three years, still results in losses of 10 to 15%. Also other important crop plants like wheat, radish, maize and barley suffer from this effect.

Specific strains of the Pseudonomas fluorescens /putida group have been used as seed inoculants on crop plants to promote growth and increase yields (Kloepper et al., 1980). These pseudomonads, termed plant growth-promoting rhizobacteria (PGPR), rapidly colonize plant roots of potato, sugar beet and radish and cause statistically significant yield increases up to 144% in field tests. Crop yield increase and growth stimulation can only be applied to short rotation soils since the bacteria have no beneficial effect on healthy plants in long rotation soils. Successful strains are antagonistic known gram-positive and gram-negative pathogenic against bacteria (Erwinia carotovora var. carotovora, E.carotovora var. atroseptica, Streptomyces scabies) and fungi (Fusarium Verticilium albo-atrum, tabacinum, Rhizoctonia solani, Alternaria solani, Phoma exigua) as well as a number of other non-pathogenic microorganisms isolated from the rhizosphere or the soil (Geels and Schippers, 1983; de Weger et al., 1986). In 1980, Kloepper et al., carried out a study in order to

determine the nature of this antagonism to potentially deleterious rhizoplane fungi and bacteria previously observed in other studies (Burr et al., 1978; Xu and Gross, 1986; Suslow and Schroth, 1982). Evidence was presented that PGPR exert their plant growth-promoting activity by depriving native microflora of iron due to the production of extracellular siderophores which efficiently complex environmental iron, making it less available to certain native microflora. In this study, plant growth-promoting fluorescent Pseudomonas strains A1, BK1, TL3B1 and B10, isolated from potato periderm or roots, exhibited in vitro antibiosis on kings medium B agar (King et al., 1954) against the bacterium Erwinia carotovora, which causes potato soft rot and seedpiece decay. When $1\mu M$ FeCl was added to the KB plates, the antibiosis against E. carotovora did not occur, nor was the yellow-green fluorescence of PGPR produced. Antibiosis was also exhibited in vitro against E.coli K-12 AN193, which does not produce its native siderophore, enterobactin, but not against its enterobactin-producing parent, AN194. These results suggest that the fluorescent siderophores produced by PGPR under iron-deficient conditions are responsible for the antibiosis against E.carotovora and E.coli AN193.

The effect of iron on plant growth-promoting activity by PGPR in soils was also investigated. It was found that when potato seedpieces were dipped into PGPR suspensions (10⁹CFU/m1) immediately before planting in field soils, statistically significant increases in plant growth (measured as total plant wet weight) occured two weeks after emergence. However, when 120ml of 100μ M Fe^{III}EDTA per 600g of soil were added on alternate days soils planted with PGPR-inoculated to seedpieces, PGPR did not statistically increase growth, although the bacteria colonized the roots at a level comparable to that without iron. This lead to the proposal that a fluorescent siderophore from PGPR mimics the biological action of PGPR, and therefore plays a major role in plant growth promotion. To test this prosposal, the fluorescent pigment from strain B10 was isolated and purified. Both the pigment and its

ferric complex reversed iron starvation of strain B10 on KB plates induced by the ferric complexing agent, ethylenediamine-di-o-hydroxyphenylacetic acid (EDDA). The siderophore, pseudobactin, exhibited in vitro antibiosis against E.carotovora but ferric pseudobactin caused none. The effect of pseudobactin on plant growth and rhizoplane fungal colonization was also investigated. Both suspensions of B10 and pseudobactin at 10μ M caused significant plant growth increases while ferric pseudobactin at 50μ M and B10 with 50μ M Fe^{III}EDTA did not. In addition, pseudobactin and B10 treatments resulted in statistically significant reductions of fungal populations on the rhizoplane.

Kloepper et al., propose the following scenario to account for the enhancement of plant growth by PGPR. Following inoculation and planting of crop seeds, PGPR rapidly colonize roots of the developing plant. As a result of a limited supply of iron in the rhizoplane, PGPR produce siderophores which sequester iron in the root zone, making it unavailable to certain rhizoplane microorganisms. These microorganisms are unable to obtain essential quantities of iron for growth either because they do not produce siderophores, comparitively produce less siderophores than those of PGPR and/or produce siderophores which have less affinity for iron than those of PGPR. The populations of these microorganisms are reduced, and a more favourable environment for root growth is created.

Certain fluorescent pseudomonads, closely related to phytopathogenic Pseudonomas syringae, belong to the genera of deleterious rhizobacteria which includes Klebsiella, Citrobacter, Flavobacterium, Achromobacter and Arthorobacter (Suslow and Schroth, 1982). In a study by Buyer and Leong in 1986 the mode of antagonism between plant growth-promoting pseudomonads deleterious fluorescent and fluorescent pseudomonads was examined. Pseudobactin B10, pseudobactin 7SR1 from sugar beet-deleterious Pseudomonas 7SR1 and pseudobactin A214 from bran-deleterious Pseudomonas A214 were used to determine antagonism between strains. Three patterns of results were observed. Pattern 1: strain A214 on KB plates inhibited

strain B10, while the converse was not true. Strain B10 could not utilize pseudobactin A214 as a siderophore but A214 could use pseudobactin B10 or ferric pseudobactin for iron transport. Pattern 2: Results showed that when either strain B10 or 7SR1 was established first, the established strain inhibited the growth of the other. Each siderophore inhibited the growth of the other strain and strains B10 and 7SR1 could not use each other's siderophore. Pattern 3: Results showed that A214 did not inhibit strain 7SR1 and vice-versa. Each strain was not inhibited by, and could use, the other's siderophore. From this study the authors conclude that growth inhibition of certain fluorescent *Pseudomonas* strains by specific beneficial fluorescent Pseudomonas strains is due in part to the inability of susceptible strains to utilize the siderophores from beneficial strains, since they lack the corresponding outer-membrane receptor. Conversely, deleterious strains which are able to use the siderophore from a beneficial strain will be resistant. It is conceivable therefore, that following inoculation of a crop with a beneficial strain, the rhizosphere population density of any microorganism that can use the beneficial strain's siderophore will actually increase. Where natural populations of beneficial and deleterious strains cannot use each other's siderophores, resulting in mutual antagonism, factors such as the relative binding constants for iron (iii) of their siderophores, the amount of siderophore production and the efficiency of the iron assimilation systems of each strain might determine which species predominates in an iron-limiting environment. Other modes of antagonism besides iron deprivation of deleterious microorganisms could contribute to plant growth promotion by beneficial strains. In this study, antagonism between some strains occurred even in the presence of iron (III), implicating antibiotics or bacteriocins in a second mode of action.

In a study by de Weger *et al.*, in 1988, *Pseudomonas putida* WCS358 was shown to be able to take up Fe^{3+} complexed to the siderophore of another plant-beneficial *P.fluorescens* strain, WCS374. Pathogenic rhizobacteria and rhizofungi tested were

neither able to grow on Fe³⁺-deficient medium in the presence ⁵⁵Fe³⁺ of pseudobactin 358 nor able to take up from ⁵⁵Fe³⁺-pseudobactin 358. The results indicate that competition for Fe³⁺, at the level of uptake of Fe³⁺ from Fe³⁺-siderophore complexes, is the basis for the action of P. putida WCS358 as a microbial pesticide. The authors suggest that results of antibiosis assays obtained on different media (e.g. King B medium and standard succinate medium) are much more variable than the results of uptake experiments and suggest that conclusions about the ability of a strain to antagonize other microorganisms by virtue of its siderophore are more accurate when based on antibiosis assays in combination with Fe³⁺ uptake experiments. The recent finding of a fluorescent Pseudomonas strain whose antibiotic activity towards fungal growth is due to an antibiotic which is only active under low iron conditions supports this suggestion (Gill and Warren, 1988).

1.6.2 Genetic analysis of iron assimilation systems in pseudomonads.

To understand the relationship between growth stimulation and the production of siderophore, knowledge of the organization and regulation of siderophore biosynthesis is needed. Marugg et al.(1985), isolated mutants defective in the biosynthesis of pseudobactin 358 after mutagenesis with transposon Tn5. Complementation of these mutants with cosmid clones of a genomic library of WCS358 resulted in the identification of involved five separate gene clusters in siderophore biosynthesis. A major gene cluster, cluster A, measuring at least 33.5kb has been identified as being required for synthesis of the siderophore. The genetic information continues beyond one end of the 33.5kb region, as two mutants with insertions located near this end could not be complemented by any of the cosmids or subclones. The other end of the region was extended by 18kb by the isolation of an overlapping cosmid clone from a WCS358 genomic library. Besides the biosynthetic genes, the overlap also contained DNA information for the uptake and utilization of pseudobactin 358.

Analysis of the transcriptional organization of cluster A revealed the presence of at least five transcriptional units in the region.

of transcriptional units Some the are extremely long; transcriptional unit II seems to cover the EcoRl fragment of 13.5kb entirely, while transcriptional unit IV seems to be at least 5kb. It is not clear whether these transcriptional units form large open reading frames or consist of operon-like structures with multiple genes. By RNA-RNA hybridization, using RNA isolated from cultures of wild-type WCS358 grown under iron-rich and limited conditions, it was demonstrated that the expression of some of the genes within cluster A was regulated by iron at the transcriptional level.

In this study, the authors were unable to assign biosynthetic functions to the cluster A. However, genes in mutant siderophores from three of the Tn5 induced mutants were purified and their amino acid composition determined. The analysis demonstrated that all three produce the complete wild-type peptide. This indicates, therefore, that the mutants of cluster A are probably defective in certain steps in the synthesis of the hydroxyquinoline-derived group. Presumably the genes for the peptide lie on one of the other gene clusters identified as being necessary for siderophore biosynthesis in WCS358.

Using a gene bank of Pseudomonas sp. strain B10 DNA and one hundred and fifty four non-flourescent mutants defective in the production of pseudobactin, Morres et al.(1984), identified eight different recombinant cosmids carrying pseudobactin biosynthetic genes. Although the total number of genes involved biosynthetic in the pathway is not yet known, the complementation pattern suggests that a minimum of twelve genes are needed. Results indicated that at least four gene clusters are involved in the biosynthesis of pseudobactin compared with a single gene cluster involved in the biosynthesis of either enterobactin or aerobactin. This seems reasonable considering the structural complexity of pseudobactin.

Magazin et al. (1986), in a subsequent study, cloned the gene

coding for the outer membrane receptor protein for ferric pseudobactin. Pseudomonas A124 and A225 were complemented for the ability to utilize pseudobactin as a siderophore by mating the gene bank of DNA from Pseudomonas B10 en masse using the helper plasmid pRK2013. Pseudobactin-resistant transconjugants were selected on antibiotic-containing plates supplemented with pseudobactin. A recombinant cosmid pJLM300 was subjected to Tn5 mutagenesis and the functional ferric pseudobactin gene located to a region of approximately 2.4Kb which is consistent with the molecular weight of 85,000 Da for the outer membrane receptor. Mobilization of pJLM300 into Pseudomonas A124 and A225, the growth of which is inhibited by *Pseudomonas* B10 or pseudobactin, rendered these strains no longer susceptible to iron starvation because they were now able to transport ferric pseudobactin. Transposon Tn5 insertion mutants of Pseudomonas B10 lacking the receptor protein were generated by a marker exchange technique and were found to be defective in ferric pseudobactin transport. The gene for the outer membrane receptor was found to be closely associated with siderophore biosynthesis genes. They were found to flank the receptor gene on both sides and were on separate operons. The production of pseudobactin, the receptor protein and four other outer membrane proteins in Pseudomonas B10 was coordinately regulated by the level of intracellular iron.

O'Sullivan et al.(1990), isolated twenty two Tn5 induced siderophore of mutants Pseudomonas strain M114. Five complementing cosmid clones were isolated from a pLAFR1 based gene bank of the parent strain and the complementation pattern indicated that a minimum of five siderophore biosynthesis genes were encoded on the clones. One clone was also found to carry the coding gene for the outer membrane receptor for pseudobactin M114. The localization, on this clone, of a fur-like regulatory gene was also determined. It was shown that the clone, pMS639, complemented the siderophore regulatory mutant M114FR2 isolated in a previous study (O'Sullivan and O'Gara, 1990). The genetic locus encoding the outer membrane receptor for pseudobactin M114 was situated at a distance of

7.3Kb from the regulatory region. The results in this study indicated that genes controlling siderophore biosynthesis, the iron-siderophore receptor, as well as the *fur*-like repressor of the iron uptake system in strain Mll4 are clustered within the 27.2Kb insert of the clone pMS639.

By studying the genetics of iron uptake by these plant growth-promoting pseudomonads it is hoped that these iron introduced sequestering systems into may be other plant-beneficial bacteria their to increase iron competitiveness in the rhizosphere. The full potential of rhizobacteria and other microorganisms to promote plant growth will be realised only when there is a better understanding of the factors affecting their ecology and establishment on roots. This will require the cooperative efforts of bacterial ecologists, plant pathologists, physiologists, biochemists and genetic engineers, in order to determine the important factors which enable a microorganism to successfully compete in a particular ecological niche. The competitive ability of epiphytic bacteria to colonize roots and their capacity to exclude deleterious microorganisms from the root surface could be greatly improved through genetic engineering to enable them to tolerate moisture stress or to produce a wider array of metabolites affecting a greater spectrum of deleterious microorganisms. Characters that allow rhizobacteria to proliferate on roots are the key to using other beneficial bacteria as root colonizers.

1.6.3 Siderophores in Rhizobium species.

The rhizobia, another species of plant beneficial bacteria, are soil microorganisms that can interact with leguminous plants to form root nodules within which conditions are favourable for bacterial nitrogen fixation. Siderophore production by rhizobia has been of particular interest in view of the prominent role of iron enzymes at several stages in the nitrogen fixation and assimilation process. The iron enzymes and proteins involved include ferredoxin, hydrogenase, nitrogenase and leghaemoglobin. Nitrogenase, consisting of two proteins and having at least 30 iron atoms, can constitute 10 to 12% of the total protein in a bacterial cell and leghaemoglobin may represent as much as 25 to 30% of the total soluble protein in infected plant cells (Verma and Long, 1983). The synthesis of these and other iron-containing enzymes requires that both plant and bacteria have an adequate supply of iron. A brief overview of the rhizobium-legume symbiosis will be given before discussing the role of siderophores in this process.

1.6.4 The Rhizobium-legume symbiosis.

Infection of legume roots normally takes place through the root hairs which become elongated deformed and curled (Yao and Vincent,1983). Rhizobia are differentiated by their host species.

Rhizobium-Plant Associations.

Rhizobium meliloti		Alfalfa		
Rhizobium	leguminosarum			
	biovar viciae	Pea, vetch		
	biovar trifolii	Clover		
	biovar <i>phaseoli</i>	Bean		
Rhizobium fredii		Soybean		
Bradyrhizobium japonicum		Soybean		
Rhizobium loti		Lotus		
Azorhizobium caulinodans		Sesbania (stem)		
Bradyrhizobium spp.		Parasponia (non-legume)		

Rhizobium are chemotactic towards plant roots and when host plants are present rhizobia are stimulated, resulting in the occurence of large populations in the rhizosphere (up to 10^5 to 10^7 cells per gram of soil). As root hair curling takes place, the cells of the root cortex under the epidermis begin dividing (Libbenga and Harkes, 1973). Bacteria trapped in a curled root hair, or between a hair and another cell, proliferate and begin to infect the outer plant cells. As this happens, the invaded plant cell is stimulated to produce a cell wall sheath or infection thread (Callaham and Torrey, 1981). The body of the nodule is established in the plant root by cell divisions and the infection threads spread through and penetrate individual target cells within the nodule. Bacteria are released into the plant cytoplasm itself and cells divide repeatedly until a mature nodule is formed. The bacteria, once liberated from the infection thread, multiply rapidly and become swollen and irregular in shape, forming bacteroids enveloped in plant plasma membranes (Robertson *et al.*, 1978). It is at this stage that symbiotic nitrogen fixation and metabolite exchange occurs.

In various *Rhizobium* species, such as *R.leguminosarum* and *R.meliloti*, common and host-specific nodulation (nod) genes have been identified which determine infection and nodulation of specific hosts (Long, 1989) (Fig.1.11).

Rhizobium meliloti	D_1	L ABCIJ		Q	, G	EF	<u> </u>
		"cc	immon"	host	specific		
Rhizobium leguminosarum	NM	L	EF	D	ABCIJ		x
	host	host specific			"common"		

Most nodulation genes are induced when cells are exposed to plant exudates or extracts with the exception of the *nodD* gene which is constitutive (Mulligan and Long, 1985; Innes *et al.*, 1985). This induction depends on the *nodD* gene product. In several plant-rhizobium systems, the inducing molecules have been purified from plant exudates and identified as flavonoids, three ring aromatic compounds derived from phenylpropanoid metabolism. In alfalfa and clover, the most active inducers are flavones, such as luteolin (3,'4',5,7-tetrahydroxyflavone) (Peters *et al.*, 1986) and DHF (7,4'-dihydroxyflavone) (Redmond *et al.*, 1986). In soybean, the natural inducers include an

isoflavone, daidzein (Kosslak *et al.*, 1987). Rhizobium cells induced to express *nod*ABC export a factor that affects root morphology and root hair growth (van Brussel *et al.*, 1986). Recently, using *R.meliloti* strains overproducing symbiotic Nod factors, the major alfalfa-specific signal, Nod Rm-1, was isolated from filtrates of *R.meliloti* cultures after induction of *nod* gene transcription by luteolin (Lerouge *et al.*, 1990). Nod Rm-1 was shown to be a sulphated β -1,4-tetrasaccharide of D-glucosamine in which three amino groups are acetylated and one acylated with a C bis-unsaturated fatty acid. This purified Nod signal specifically elicited root hair deformation on the alfalfa host plant.

After infection is initiated, the bacteria must penetrate and be released into host cells. This requires correct bacterial as well as plant surface components. The specific bacterial components involved are the extracellular polysaccharides which include charged heteropolysaccharides, neutral β -glucans, and lipopolysaccharides (Carlson et al., 1987, 1988). Genetic evidence for polysaccharide involvement in infection is very strong (Leigh et al., 1987; Muller et al., 1988; Noel et al., 1986; Carlson et al., 1987) and genetic studies should make it possible to identify which polysaccharides are involved in the Rhizobium symbioses. Possible roles for extracellular polysaccharides are, signals or substrates for signal production, osmotic materials necessary during invasion and recognition factors that act to present and/or to disguise the bacterium during invasion. The plant surfaces, cell wall and other secretions as well as the underlying plant membrane, are also implicated in the correct invasion and differentiation of bacteria into plants. The inside of the infection thread is packed with bacteria and a matrix, one component of which is a plant-derived glycoprotein (Bradley et al., 1988). Due to the intimate contact occuring between this protein and the invading bacteria, it may be a key element in the recognition process. Genes for nitrogen fixation in Rhizobium are divided into two groups: nif genes are those with homologs in free-living nitrogen fixation systems such as Klebsiella while fix genes

are those genes required for symbiotic nitrogen fixation but whose function is not analogous to any free-living function.

In Klebsiella pneumoniae transcription of the nitrogen fixation genes is regulated by the NifL and NifA proteins which are encoded by the nif LA operon (Gussin et al., 1986). NifA is a transcriptional activator which binds to specific sequences upstream of nif promoters to positively control nif transcription under anaerobic, nitrogen limiting conditions. NifL is a negative regulator which responds to increasing concentrations of fixed nitrogen or oxygen to antagonise the action of NifA (Hill et al., 1981) thus preventing synthesis of nitrogenase under inappropriate conditions. In rhizobia, a NifL equivalent has not been identified, and NifA appears to be directly responsive to oxygen (Albright et al., 1988; Fischer and Hennecke, 1987). The nitrogen enzyme is irreversibly inactivated by oxygen, so preventing oxygen damage is a physiological challenge to the nitrogen fixing organism. This challenge is met by the presence within nodules of the red pigment leghaemoglobin. Leghaemoglobin proteins bind oxygen then release it when the local concentration drops below a certain level thus providing a high flux for the Rhizobium to use in respiration but in an environment with low free oxygen (Appleby, 1984). The bacteria face an additional challenge of generating enough ATP to satisfy the requirement for it during nitrogenase function. To this end the plant supplies carbon compounds, derived from photosynthesis in the shoot, to the bacteria for generation of ATP and reduced electrons. Finally, the plant itself assimilates the primary fixation product (ammonia) into glutamine and other amino acids.

1.6.5. Siderophores and Symbiosis.

What role, if any, do siderophores play in the Rhizobium-legume symbiosis? To date, this question remains unanswered.

That host plants and invading rhizobia must have an adequate supply of iron for the synthesis of nitrogenase, hydrogenase, ferredoxin, leghaemoglobin and other iron-containing compounds is well illustrated by the report that iron deficiency specifically limits nodule development in peanut plants inoculated with Bradyrhizobium sp. (O'Hara and Dilworth, 1988). Severely iron-deficient peanuts (Arachis hypogaea L.) grown on calcareous soils in Central Thailand failed to nodulate until given foliar iron applications. Nodule initiation in peanut was not affected by iron-deficiency as the production of numerous nodule initials on the taproot of peanut seedlings growing under conditions which limited nodule development showed that the bradyrhizobia were able to infect root tissues and invade root cells before iron deficiency limited symbiotic development. The most sensitive stage of the peanut symbiosis to iron-deficiency was early nodule development subsequent to nodule initiation. The primary limitation appeared to occur on bradyrhizobial proliferation and subsequent bacteroid development. There were markedly fewer bacteroids present in root nodules on iron-deficient plants than in nodules formed on plants supplied with foliar applied iron. Leghaemoglobin synthesis was also decreased in nodules formed on iron-stressed peanuts.

The mobility of foliar applied iron has been shown in a number of plant species (Brown *et al.*, 1965), with a rapid translocation via the phloem of foliar applied iron to actively growing regions including root and shoot apices. In this study, the results show that the rapid response in nodule development following foliar iron application is as a result of an increased iron supply the nodule initials to and the bradyrhizobia present in the nodule tissues. Rapid bacterial multiplication following the increased iron supply resulted in bacteroid development.

After infecting root tissues, rhizobia are subjected to the iron concentrations present in root tissues. Although iron availability in the soil appeared to be adequate for bradyrhizobial growth, as the growth and survival of this species was not limited in the calcareous soil in which the peanut seedlings developed iron-deficiency, its availability within the roots was not. The limitation of nodule development

by iron-deficiency is a consequence, therefore, of the plants inability to provide sufficient iron to the internal sites of bradyrhizobial proliferation at a rate adequate for normal cell division. In this study, greater sensitivity to iron stress was shown by cultivar Tainan 9 than by cultivar Robut 33-1, and symbiotic development in cv. Tainan 9 responded more than that of cv. Robut 33-1 to alleviation of iron-deficiency, indicating that at the plant cultivar level there may be significant differences in the ability of the host plant to supply iron to the rhizobia contained within developing root nodules.

Additional evidence for the importance of iron in nitrogen-fixing symbioses is provided by a mutant of Rhizobium leguminosarum which forms white, ineffective nodules on peas and has an apparent defect in iron acquisition (Nadler et al., 1990). Approximately 12,000 survivors of an N-methyl-N'-nitro-N-nitrosoquanidine -mutagenised culture of the effective strain 1062 were examined in an effort to isolate hemin-requiring mutants of R.leguminosarum. One colony, strain 116, emitted a pinkish-rose fluorescence when plates bearing this strain were excited by long-wavelength UV light. It was initially presumed that 116 accumulated the pink-fluorescing precursors of haem due to a partial block in haem biosynthesis and since similar porphyrin-accumulating mutants had been described in E.coli, (Cox and Charles, 1973), the mutation in 116 was designated as pop-1. Examination of the growth of 116 under iron replete and iron deficient conditions showed that strain 116 grew poorly in low iron broth but grew as well as 1062 in an iron-replete medium. Supplementation of the low iron broth with $122\mu M$ FeCl resulted in normal growth of 116. Iron chelating agents such as 8-hydroxyquinoline, nitrilotriacetate and N-hydroxyethylethylenediamine -triacetate were less effective than 2,2'-dipyridyl in discriminating between 116 and 1062. It was found that citrate, dihydroxybenzoate and low concentrations of 8-hydroxyquinoline restored growth of 116, while, anthranilic acid, the native siderophore, inhibited growth.

The results indicate that 116 retains the general ability to

utilize Fe (iii) solubilized by diverse chelators such as citrate and dihydroxybenzoate but may be defective in a cell-bound element of a specific iron transport system. In this study, the authors were unable to demonstrate in several bioassays that strain 116 is defective in biosynthesis of a siderophore. The authors propose that 116 is defective in iron anthranilate metabolism but whether the missing cell-bound element is thought to be the outer membrane receptor for anthranilate is unclear. No direct determinations of ⁵⁵Fe³⁺-anthranilate uptake rates, essential for this conclusion presented. The symbiotic properties were of 116 were particularly interesting. The white-green colour of 116 nodules idicated that little leghaemoglobin was accumulated in these nodules. Furthermore, no symbiotic nitrogenase activity was detected. Nodule cortical cells were filled with vesicles containing large, normal appearing bacteroids and no degeneration of the organelles of the infected cells or of the bacteroids was observed. The addition of iron citrate or FeCl to the plant growth medium at up to 10-fold the usual concentration had no effect on the nitrogenase activity of 116 nodules. The observations indicate that pop-1 arrests nodule development at a late morphological stage, after bacteroid development and that 116 is defective in the iron uptake system utilized in pea root nodule symbiosis.

Two sets of observations indicate that pop-1 causes both the defect in iron acquisition and the symbiotic effect. R plasmid-mediated mapping studies failed to obtain recombinants in which the two mutant phenotypes were separated genetically. Secondly, cosmid pKN1 from a pLAFRI based gene bank of *R.leguminosarum* B155 conferred on 116 the ability to grow on iron-limiting medium as well as functional symbiotic nitrogen fixation. This indicates that if 116 contains two closely linked mutations they are within about 24kb of each other since this is the size of the cloned insert in pKN1.

In contrast to the previous report whereby iron-deficiency *in planta* limits nodule development in peanut inoculated with *Bradyrhizobium* sp., in this report it is the inability of the
invading microorganism to assimilate iron which arrests nodule development. Presumably the plants used to detect the symbiotic effectiveness of strain 116 were not themselves iron-deficient. Thus nodule development proceeded from initiation through to bacteroid development as was the case when the iron deficiency in peanuts was corrected with foliar applications of iron. This would seem to indicate that it is in the bacteroidal state that specific (possibly different from the external) a iron assimilation system is needed for the onset of nitrogen fixation. Verma and Nadler (1984) estimate a lower limit for the iron concentration of soybean bacteroids at 0.5mM, based solely on the iron content and turnover number of the iron-containing enzyme complex dinitrogenase-dinitrogenase reductase, the relative bacteroid volume of root nodules and the diurnal nitrogen fixation activity of root nodules. When the iron importation necessary for the synthesis of other rhizobial iron and haem proteins along with the demand for iron that is required for the synthesis of the haem of the plant protein leghaemoglobin is included in this estimate, it is clear that a substantial iron flux into bacteroids is essential for symbiotic nitrogen fixation. The in planta results of the two reports described complement previous work on the effects of iron deficiency on free-living rhizobia (Roessler and Nadler, 1982). The effects of iron deficiency on haem biosynthesis in Rhizobium japonicum were examined. Tron deficient cells had a decreased maximum cell yield and a decreased cytochrome content and excreted protoporphyrin into the growth medium. The activities of the first two enzymes of haem biosynthesis, δ -aminolevulinic acid synthase and δ -aminolevulinic acid dehydrase were diminished in iron-deficient cells, but were returned to normal levels upon addition of iron to the cultures. The results of this study too suggest, that rhizobial haem synthesis in the legume root nodule may be affected by the release of iron from the host plant to the bacteroids.

In addition to these reports, there is now evidence that iron plays a regulatory role in the nitrogen fixation process as

well as playing a central role as a component of compounds such as haem and nitrogenase. In a study by Fischer et al., 1988, the amino acid sequence of the Bradyrhizobium japonicum nitrogen fixation regulatory protein NifA was aligned to the corresponding protein sequences from Klebsiella pneumoniae, Rhizobium meliloti and Rhizobium leguminosarum biovar viciae. Between the central and the DNA binding domains, an interdomain linker region was identified which was conserved in all rhizobial species but missing in the K.pneumoniae NifA protein. Two conserved cysteine residues in this region, once changed to serine residues by oligonucleotide directed mutagenesis, resulted in absolutely inactive NifA mutant proteins. In the interdomain linker region of the R.meliloti NifA protein, the sequence Cys-X₄-Cys-X₉-His-Cys-X-His perfectly fulfills the criteria by which Berg (1986) defined a potential metal-binding site in a variety of nucleic acid binding proteins. In the corresponding B. japonicum sequence some of the amino acids are replaced by tyrosine or lysine which could also serve as metal binding coordinates.

Experimental results show that the in vivo activation of a nifD'-lacZ fusion in E.coli by the B.japonicum NifA protein, but not by the K.pneumoniae NifA protein, was strongly sensitive to the addition of > 3mM EDTA to the growth medium. After the addition of Me(ii) (Me=metal) to the medium, the inhibitory effect of EDTA was overcome either fully by Fe(ii) or partly by Cd(ii), Cu(ii), Mg(ii), Mn(ii), Ni(ii) or Zn(ii). Using the chelator o-phenanthroline (which binds ferrous iron) it was found that a concentration as low as 0.03mM strongly inhibited NIFA activity in medium without added FeSO, but not in normal medium containing iron. The results suggest that the formation of active rhizobial NifA proteins requires the presence of divalent metal ions. It was also shown that the in vivo activities of the NifA proteins of B. japonicum and to a lesser extent, also of R.meliloti, but not of K.pneumoniae, are sensitive to oxygen.

A working hypothesis was proposed to provide a connection between these observations. A mechanistic model is conceivable

in which a metal (e.g. Fe(ii)) would bind in its reduced state to the interdomain linker thus aiding the correct positioning of the DNA binding domain in order to form an active NifA protein. Under aerobic conditions the metal ion may be oxidised and unable to bind to NifA with the result that the protein is now inactive. Furthermore, it may be possible that this metal is more firmly bound to the R.meliloti NifA protein than to the B. japonicum NifA protein because the activity of the former is less sensitive to both oxygen and chelators. A similarity to the ferric uptake regulation protein of E.coli, Fur, was made by the authors. Fur has been purified and shown to regulate aerobactin operon expression in vitro in the presence of Fe(ii) but not Fe(iii). Most interesting was the observation that anaerobic conditions were necessary to demonstrate this regulation. Another comparison was made to the E.coli Fnr protein which positively regulates a number of anaerobically expressed genes. The protein contains the potential redox active sequence Cys-X_-His-Cys-X_-Cys (Unden and Guest, 1985). The protein is inactivated by mutagenesis of the second cysteine to serine and the activity of Fnr appears to be iron dependent in vivo.

Similarily, it was recently shown (Henderson *et al.*, 1989) that the ability of the *Klebsiella pneumoniae nifL* gene product to antagonise NifA mediated transcriptional activation from the *nifH* promoter *in vivo* was inhibited either by metal deprivation or by the presence of the iron chelators EDDA or Desferal in the growth medium. Inhibition of the repressive activity of NifL was reversed by the addition of ferrous or manganous ions to the medium but was unaffected by other transition metals.

The evidence presented thus far demonstrates that iron plays a key role in symbiotic nitrogen fixation. The involvement of siderophores in this process has yet to be demonstrated but knowledge of how rhizobia acquire iron should help further understanding of this important symbiosis.

1.6.6 Siderophores produced by Rhizobium species.

The large quantities of iron utilized in the Rhizobium-legume symbiosis prompted the investigation of the high affinity iron transport system of *Rhizobium meliloti* but to date no role for the external siderophore in symbiotic iron acquisition by *Rhizobium meliloti* or any other species tested, has been described.

Once the colourimetric assays for catechol and hydroxamate had been replaced with an equally sensitive yet less restrictive bacterial iron nutrition bioassay, it became possible to detect and isolate Rhizobactin, the atypical siderophore of Rhizobium meliloti DM4 (Smith and Neilands, 1984). Based on the supposition that a functional high affinity iron transport system would be essential for the Rhizobium-legume symbiosis, bacterial iron nutrition bioassays were performed on several additional isolates of R. meliloti. Only six of the thirteen isolates tested were stimulated by rhizobactin. The result was unanticipated because it indicated that a rhizobactin-based iron transport was non-functional in several other R.meliloti strains, but considering the strain specificity displayed by many *Pseudomonas* strains with regard to production and uptake of siderophores, this result is not unusual.

In a subsequent study (Smith *et al.*, 1985), the structure of rhizobactin was elucidated and presented as an aminopoly carboxylic acid, thus extending the types of microbial siderophores to three distinct classes. Rhizobactin, N^2 -[2-[(1-carboxyethyl)amino]ethyl]- N^6 -(3-carboxy-3-hydroxy

-1-oxopropyl)lysine, contains ethylenediamine-dicarboxyl and α -hydroxycarboxyl moieties as metal coordinating ligands, the ethylenediamine group being novel as a natural product. The α -hydroxycarboxylate ligand of rhizobactin, present as a malamic acid substituent, has been found in two additional types of siderophore. Three members of a mixed catechol-hydroxamate type contain β -hydroxyaspartic acid (Yang and Leong, 1984).It is also present as citrate in the three citrate-hydroxamate type siderophores.

Besides the microbial products, five homologues of amino acids

from graminaceous plants, regarded as possible phytosiderophores, contain α -hydroxycarboxylate and α -amino-carboxylate ligands. A sixth homologue, containing three amino-carboxylate ligands has been isolated from diverse vascular plants (Budesinsky et al., 1980). Among such plants is alfalfa, a symbiotic host of the bacterium which excretes rhizobactin. The phytosiderophores display strong coordination properties toward dissimilar divalent and trivalent metals (Sugiura et al., 1981). Rhizobactin's similar features may modify the notion that siderophores contain virtually ferric-specific ligands and the biological function of these compounds may extend beyond iron transport. Smith and Neilands (1984) note that a strain specific synthesis/utilization of unusual amino/imino carboxylic acids, termed opines, produced by plant cells transformed by Agrobacterium spp., may prove to be of general reference to bacteria within the Rhizobiaceae family. In a subsequent report Smith and Neilands (1987) discuss the structure of rhizobactin in the context of it providing a new biochemical activity for an N²-substituted amino acid, namely iron assimilation. Due to the structural relatedness of rhizobactin and the opines, the strain-specific patterns of rhizobactin and opine synthesis/utilization, and the chelating ability of these \mathbb{N} -substituted amino acids, the authors conclude that rhizobactin is biochemically related to the opines, suggesting that some opines are capable of iron transport. As defined by Tempé et al. (1982), opines are " substances synthesized by the cells of the host plant in response to a stimulus of the pathogen. Their presence creates favourable environmental conditions for the pathogen and contribute to its dissemination".

Agrobacterium tumefaciens strains B6 and a B6 derivative cured of pTiB6806 (A217) produce a phenolate-type siderophore, agrobactin. It is worth noting that virtually all mutants (17 out of 18) of A217 which were defective in the synthesis of agrobactin, retained their virulent phenotype on sunflower plants and on carrot root discs after pTiB6806 was reintroduced (Leong and Neilands, 1981). Chromatographic analyses and

bioassays failed to demonstrate the presence of agrobactin in plant tissue infected with A.tumefaciens B6. Given the importance of iron in microbial metabolism, it is likely that agrobactin-producing strains of Agrobacterium synthesize or utilize additional siderophores under particular growth regimes. Experimental evidence indicates that opines could fullfill this role. The situation with respect to Rhizobium spp. appears to be similar in that no role for a free-living siderophore in symbiosis has been described yet it has been clearly demonstrated that iron is absolutely necessary for this process. That a specific uptake system for iron may exist in bacteroids has been suggested by the work carried out by Nadler et al.,(1990) (already discussed), but how this iron is supplied to the bacteroids remains to be determined.

1.6.7 Genetic analysis of rhizobactin production.

The development of a Universal Chemical Assay for the detection of siderophores based solely on their ability to chelate iron and not on structure, paved the way for easy detection of siderophores such as the atypical rhizobactin (Schwyn and Neilands, 1987). The assay has also proved extremely useful for investigation of the molecular genetics of siderophore systems.

Gill and Neilands (1989) reported on the cloning of a genomic region required for a high-affinity iron-uptake system in *Rhizobium meliloti* 1021. Using the Chrome Azurol S universal chemical assay, a collection of transposon induced mutants of *Rhizobium meliloti* 1021 defective in siderophore-mediated iron assimilation were obtained and classified as biosynthetic, transport, or regulatory. The assay depends on a blue dye (a ternary complex of chrome azurol S, a detergent and iron) serving as an indicator. When a strong chelator removes iron from the dye it turns from blue to orange. On agar plates, mutants exhibiting no orange halo and anable to grow on iron chelator medium were classified as biosynthetic, Rzb⁻, mutants producing large haloes and anable to grow on iron chelator medium were classified as defective in the uptake of the ferric rhizobactin 1021 complex, Rbu^{*}, while mutants producing a large halo but able to grow on iron chelator medium or medium containing high iron concentrations were classified as defective in a negative regulatory function for rhizobactin 1021,Rbr^{*}. The Rbu^{*} class of mutants were not investigated because none of the mutants of the three classes isolated was defective in the production of any of the low-iron-induced outer membrane proteins ,suggesting that a ferric-siderophore outer membrane receptor protein was not encoded by a gene requiring the expression of siderophore production genes and was not the site of the Rbu^{*} mutations.

Using a cosmid library of *R.meliloti* 1021 all of the Rzb mutants were complemented and the site of the Rbr mutations localized within a single genomic region, indicating that the genes required for siderophore biosynthesis and possibly its regulation are clustered in the genome. Complete characterization of the structure of rhizobactin 1021 and identification of the genes required for its biosynthesis remain to be determined in order to establish that all of the biosynthetic genes have been isolated in this study.

Nodulation tests performed on a number of mutants showed that nodules were formed by mutants representative of every class using standard nodulation medium. Adjusting the iron content of the medium to a much lower level did not inhibit nodule formation although nodules were smaller, less coloured and the plants exhibited some chlorsis. Because the nodules contained viable bacteria it was concluded that the rhizobial iron assimilation system serves no obvious role in nodulation or bacteroid development.

As in the case of *Pseudomonas* spp., the rhizobial high-affinity iron-uptake system may function in the competition among soil microbes for access to available iron and may enhance survival of the free-living form. Carillo and Peralta (1988) report siderophore-like activities in *Rhizobium phaseoli*. Nineteen out of fifty-two strains of *R.phaseoli* showed siderophore-like activity when bioassayed against *Xanthomonas campestris*

pv.phaseoli CBP123 and Pseudomonas syringae pv.phaseolicola (Psp) in low iron medium. Whereas ten of the supernatants inhibited both pathogens, one strain, *R.phaseoli* CPMEX preferentially inhibited Psp while R.phaseoli CPMEX 26 inhibited Xanthomonas campestris. It was also demonstrated that the twenty-fold concentrated spent media from two R.phaseoli strains diminished the necrotic and chlorotic damage caused by CBP123 and Psp on the leaves of plants of P.vulgaris in the greenhouse. This report may indicate that the role of external in siderophores Rhizobium is similar to the role of siderophores in Pseudomonas spp., i.e., assimilation of iron thereby depriving pathogenic bacteria and other competing bacteria present in the rhizosphere. A situation could be invisaged whereby one *Rhizobium* strain producing a siderophore could outcompete another Rhizobium strain not producing a siderophore or producing one with less affinity for iron. It has already been shown that R.meliloti DM4 and R.meliloti 1021 produce different siderophores which each stimulate the growth of the source organism, but which antagonize the growth of the other (Gill and Neilands, 1989).

Other siderophores isolated from *Rhizobium* species include a catechol-like siderophore from cowpea *Rhizobium* RA-1 isolated by Modi *et al.*,(1985). Colourimetric assays and thin-layer chromatography identified 2,3-DHBA as a component of the siderophore, while hydrolysis studies revealed the presence of threonine and glycine. Putel *et al.*, (1988) isolated and partially characterized a phenolate siderophore from *Rhizobium leguminosarum* IARI102. Again, 2,3-DHBA was detected as the main component with threonine as the amino acid conjugate.

Production of a siderophore by *Bradyrhizobium japonicum* 61A152 was detected by Guerinot *et al.*,(1990) using the chrome azurol S assay. Thin-layer chromatography in combination with high-voltage paper electrophoresis and a specific enzyme assay, identified the siderophore as citric acid. That ferric citrate could serve as an iron source was confirmed by growth and transport assays. This is the first report of citric acid release in response to iron stress by a rhizosphere bacterium.

A number of soil-inhabiting fungi have been shown to release both citric and malic acid, while release of organic acids in response to iron stress is well documented for Neurospora crassa (Winkelmann, 1979). The finding that only one of twenty strains tested produced citric acid adds to the growing evidence that rhizobia exhibit strain-to-strain variation in the ability to produce particular siderophores. Bradyrhizobia as a group may not possess highly developed iron acquisition systems, having evolved in the acid soils of the tropics, where iron is more readily available than in neutral or high pH soils. Whether the low incidence of siderophore production reported in this study turns out to be a general property of bradyrhizobial strains remains to be determined. In addition to fungi, many plants are also known to accumulate and/or release citric acid under iron stress (Landsbreg, 1981). Soybeans have been shown to transport iron as ferric citrate, xylem fluid containing 5 to 6 μ M ferric citrate with citric acid present at 1 to 2 mM (Tiffin, 1970). Therefore it is not surprising that B. japonicum can utilize ferric citrate as an iron source since it encounters it in the rhizosphere and the plant itself. All the B. japonicum strains tested were found to be able to use ferric citrate as an iron source.

1.7 Iron acquisition in Higher Plants.

Apart from iron acquisition by bacteria and fungi in the soil, plants have also developed systems for the uptake of iron. There is no doubt that the root-soil interface (rhizosphere) plays an important role in this process. Plants adapted to soil with low iron availability (e.g. calcareous soils) have evolved various iron-deficiency-induced adaptation mechanisms to mobilize iron in the rhizosphere and to enhance uptake and translocation from root to shoot.

Plants, theoretically, can enhance iron solubility and hence availability by several ways: 1) Release of reducing compounds from the roots, 2) Release of protons which facilitate iron solubility as well as net rate of Fe³⁺ reduction, 3) Release of exudates with iron chelating character, and 4) enhanced root surface area to support the various physiological processes for iron mobilization in the rhizosphere. Higher plants do not possess each of the above mentioned adaptive mechanisms, but distinct differences exist among plant species. These differences in response to iron deficiency have been classified into two groups or strategies.

Strategy I is primarily characterized by an inducible Fe³⁺ reduction process, and in some cases by an inducible proton release. Strategy II is characterized by a release of specific chelators (phytosiderophores) for the mobilization and uptake of Fe³⁺ and is confined to the grasses.

Strategy I: Chaney *et al.*, (1972) showed that addition of ferrous chelators inhibited uptake of iron from ferric chelates by soybean. This demonstrated that ferric chelates must be reduced before uptake. It was proposed that transfer of electrons from the cytosol to the extracellular ferric compound across the plasma membrane took place. Bienfait (1988) proposed that two different plasma membrane redox systems exist in plant cells; a standard system present in all cells found capable of reducing ferricyanide only and having nothing to do with iron transport, and a Turbo system induced by iron shortage in the root epidermal cells. The function of this system is to produce ferrous ions for iron uptake. NADPH is proposed as the electron donor for the turbo reductase and its mechanism of action is presented diagramatically in Fig. (1.12



Another frequently observed characteristic of strategy I is an

ATPase-driven proton pump (Romheld *et al.*, 1984). It has been proposed that this proton pump functions to lower the pH in the rhizosphere, the enhanced acidification under iron deficiency conditions resulting in an increased solubility of inorganic Fe^{3+} compounds. The major factors affecting iron acquisition are probably the activation of both the plasma membrane-bound reductases and the release of reducing compounds.

Strategy II: Plant species that possess strategy II have two main characteristics, 1) an iron deficiency-induced release of iron mobilising compounds (phytosiderophores) and 2) а regulated membrane for transport system ferrated phytosiderophores.

Roots of grasses with iron deficiency release enhanced amounts of compounds which can solubilize inorganic iron i.e. ferric hydroxide. These root exudates or phytosiderophores, have been identified as various hydroxy and amino substituted iminocarboxylic acids. Mugineic acid and avenic acid, chemically related to nicotinamine, are two examples of phytosiderophores (Takagi et al., 1984). Romheld et al., (1987) showed that as a consequence of the enhanced release of phytosiderophores by iron deficient barley plants, the uptake rate of iron supplied as sparingly soluble ferric hydroxide is about 100 times higher compared to plants with sufficient iron. In addition, the uptake rate of iron by barley plants is about 80 times higher than that for iron deficient potato plants which is consistent with the fact that potato plants do not release phytosiderophores. Rates of iron uptake by barley roots supplied with ferrated phytosiderophores are about 1000 times higher than synthetic (FeEDDHA) or microbial (ferrioxamine) chelates. Thus. 1-10nM iron supplied ferrated iron as phytosiderophore is sufficient for optimal growth of barley and sorghum grown in long term experiments in nutrient solution, whereas 10µM FeEDDHA or ferrioxamine are normally required for growth of these plants. The low concentration of ferrated phytosiderophore required indicates a highly specific uptake system. A transport protein for iron phytosiderophores across the plasma membrane has been postulated similar to that of

microorganisms (Romheld and Marschner, 1986).

Finally, when considering iron acquisition under natural conditions, the possible interplay of plants with rhizosphere microorganisms must be considered. Both positive and negative effects can be postulated. One positive effect would be the enhancement of iron solubility by a build up of unspecific iron chelates. The number of microorganisms in the rhizosphere is increased extensively when root exudates are present and because of their fast turnover, the concentration of a wide range of organic and amino acids would also increase markedly in the rhizosphere. These compounds could mobilize sparingly soluble iron thus benefiting the plants. Another effect could be an enhancement of iron solubility due to a release of siderophores produced by microorganisms. Iron solubilized by siderophores could promote the transfer of iron to the roots. al.(1986) Jurkevitsch et reported the alleviation of lime-induced chlorosis in peanuts by Pseudomonas sp, siderophores. However, convincing evidence for the uptake mechanism of iron supplied as ferrated siderophores in higher plants is lacking. Competition between microorganisms and roots of higher plants for iron must theoretically be considered. A negative effect of microorganisms on plant iron acquisition can be invisaged due to the fact that many Pseudomonas spp., for example, form stronger Fe^{3+} chelates than the phytosiderophores released by grasses.

It has been established that many microbial siderophores are less efficient iron chelates for grasses than the plant-borne phytosiderophores (Romheld, 1987). The low accessibility of ferrated microbial siderophores such as ferrioxamine for the reducing systems of plant species exhibiting strategy I has also been described (Romheld and Marschner, 1983). Therefore, microbial siderophores as potential chelators of iron for higher plants may not be very important in general.

Although more than a hundred plant species were investigated for their mechanisms for iron acquisition, only a limited number of plant species of various systematic categories have been tested. Nevertheless, tests indicate a definite

distribution of strategies I and II among plant species, the taxonomic boundary existing between the Poales and the other orders of higher plants. Even plant species from closely related orders to Poales, such as Restionales, Eriocaulales, Commelinales, Juncales and Cyperales, possess the characteristics of strategy I. Thus the strategy II mechanism for iron acquisition appears to be confined to a single species of the monocotyledons. This therefore excludes those plant species acting as hosts to various rhizobia and certainly, species tested such as Arachis hypogea L., Glycine max., Pisum sativum L., and Vicia faba L., all exhibit the strategy I mechanism. Therefore, it is interesting to note the description by Budesinsky et al. (1980), of the production of a compound by diverse vascular plants, including alfalfa, which as а polyaminocarboxylate, is regarded as а possible phytosiderophore. Also interesting to note is the strong structural relationship between rhizobactin, the siderophore of Rhizobium meliloti DM4, and the phytosiderophores. Were it not the seemingly strict confinement of phytosiderophore for production and utilization to the Poales, it would be tempting to suggest that rhizobactin, as well as fullfilling the iron requirements of its producing bacterium, might also play a role in supplying iron to its host plant.

Rhizobium meliloti DM4 was first examined for its siderophore because it was felt that considering the large iron requirement of symbiotic nitrogen fixation, a siderophore produced by this microorganism must certainly be involved in the symbiotic process. Subsequent investigations of mutants not synthesizing the siderophore indicate that this would seem not to be the case. However it appears too great a coincidence that Rhizobium meliloti DM4 should produce an atypical siderophore bearing a strong structural relationship to compounds regarded as plant this siderophores. The role of siderophore and other siderophores produced by R.meliloti strains which seem to also to the recently discovered third class of belong siderophores, in the nitrogen-fixing symbiosis, remains to be determined.

Chapter Two Materials and Methods. 2.1 Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are described in Table 2.1 and 2.2.

Strain	Phenotype/Genotype	Source or Reference
		Pühler, A.
R.meliloti	wild isolates	University of
220-1 to 220-20	4	Bielefeld.
220-5Rif ^r	Spontaneous Rif ^r	This study.
	derivative.	
220-5Rif ^r deriv	vatives	
220-5-1	Siderophore (Tn5-mob)	н.
220-5-2		÷.
220-5-3		
220-5-4		
2011	wild type strain Nod ⁺	Rosenberg et
	Fix ⁺ on <i>Medicago sativa</i>	a. al., (1981).
2011Rif ^r	Spontaneous Rif ^r	This study
	derivative	
2011Rif ^r deriv	atives	
2011-1	Siderophore (Tn5-mob)	This study
2011-2	T.	"
2011-3		
2011-4		

Table 2.1

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Table 2.1 (contd.)
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Strain	Phenotype/Genotype	Source or reference
DM4	Nod [†] Fix ⁺ , Cm ^r	Gill, P.
	derivative of 102F28	Berkley,
		California.
102F34	Nod [†] Fix [†]	
65	wild type	D.C.U. stocks.
65c	Spontaneously cured	D.C.U. stocks.
	of a cryptic plasmid.	
R.leguminosaru	D	
biovar viciae		
VF39	Nod ⁺ Fix ⁺	
	Isolated from V.faba	University of
	nodules.	Bielefeld.
.11300	wild isolate	Hirsch P
		Rothamsted
		Exp. Station.
		·····
B151	128C53 Sm ^r , cured	
	of pRL6J1.	
B164	128C53 Sm ^r contains	D.C.U. Stocks.
	pIJ1008.	
3855	128C53 Sm ^r contains	D.C.U. Stocks.
	pRL6J1.	

Table 2.1 (contd.)

Strain	Phenotype/Genotype	Source or reference
R.leguminosa	arum	
biovar trife	olii	
1027	Nod ⁺ Fix ⁺	D.C.U. Stocks.
FYM1	wild isolate, Nod ⁺ Fix ⁺	Hirsch, P.
	metal sensitive.	Rothamsted
		Exp. Station.
FYM2	wild isolate, Nod^+Fix^+	
	metal sensitive.	
RCR 5	wild isolate	
32	n -	
46		
221	н	
R.loti		
LP22	Acid/Al tolerance	Cooper, J.,
LP27		Queen's Univ.
LP28		Belfast.
N2P2042		

Table 2.1 (c	ontd.)
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Strain	Phenotype/Genotype Sou	rce or reference
Agrobacterium		
tumefaciens		
C50	Wast met Wassi -	Maineraites of
638	vir il Ketolactose	University of
	Noc	Bielefeld
1060	contains Ri plasmid	"
4011	Vir Ti ketolactose	
	Occ Rif	
9023	Rif ^r Sm ^r derivative of C58	Hirsch, P.
	cured of both pTiC58 and	Rothamsted,
	pATC58.	Exp. Station.
Pseudomonas	wild isolate	O'Sullivan, D.
fluorescens		University
DN12		College, Cork.
Escherichia c	coli	
CSH56	F ara ∆lac pro supD	Miller, (1972)
	NalA thi	
S1 7-1	Rec derivative of 294	Simon, (1984)
	with RP,-2Tc::Mu Km::Tn7	
	in the chromosome. <i>hsd</i> R	
	pro Tp-Sm res mod ⁺ .	
JA221	F' recAl leuB6 tro∆5	Clark and
	hsdMT hsdR lacy xvl.	Carbon, (1981)

Table 2.2	Plasmids	
Plasmid	Relevant	Source or reference
	Characteristic	
pSup5011	pBR325 derivative, Tn5- <i>mob</i> Cm Tc Km	Simon, (1984).
pUC19	Ap ^r lacZ	Norrander, <i>et al.,</i> (1983).
pBR322	Ap ^r Tc ^r	Bolivar, <i>et al.,</i> (1977).
pGR1	Tn5-mob containing EcoRl fragment (9kb) from 220-5-1 cloned into pUC19.	This study

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2.3 Microbiological Media.

Solid complex media contained 15g/L Oxoid No. 3 agar. Solid minimal media contained 15g/L purified agar (Difco Laboratories). Tryptone and Yeast extract were from Oxoid. Other chemicals were from Sigma Chemical Co. LTD and BDH Chemicals LTD. All chemicals were analar grade.

All glassware used for low iron media was washed in 2M HCl and rinsed in ultra pure deionized water and all minimal and low iron media were prepared in ultra pure water. Distilled water was used to prepare complex media. Sterilization was achieved by autoclaving at 151b/in² for 20 minutes.

TY medium (Beringer, 1974)

Used for the routine culturing of fast growing *Rhizobium* strains.

Tryptone	5g
Yeast Extract	3g
CaCl ₂ .2H ₂ 0	0.7g
H ₂ 0	l litre
рН	7.2

Luria Bertani (LB) medium Used for routine culturing of *E.coli*. Tryptone 10g Yeast Extract 5g NaCl 10g

H O	1	litre
2		

pН

Chrome Azurol S medium (Schwyn and Neilands, 1987). Used to detect the production of siderophores by bacterial strains.

Pipes	30.24g
Sucrose	2g
KH ₂ PO ₄	0.03g

H₂0 pH 875ml

6.8 (with a 50% (w/w) NaOH soln.)

This mixture was autoclaved separately. After cooling , 10ml deferrated casamino acids (10%) and 10ml of a solution containing biotin, thiamine and panthothenic acid (10mg/L) were added. Also added were 10ml MgSO₄.7H₂O solution (5g/100ml) and 7.8ml CaCl₂.H₂O solution (1M). The dye solution was finally added along the glass wall with enough agitation to achieve mixing without generation of foam.

The dye solution was prepared as follows: 60.5mg CAS was dissolved in 50ml Pipes (1mM, pH 5.6) and mixed with 10ml of an iron(iii) solution (FeCl₃,10mM HCl). Under stirring this solution was slowly added to 72.9mg HDTMA dissolved in 40ml Pipes pH 5.6. The resultant dark blue liquid was autoclaved. CAS, chrome azurol s; HDTMA, hexadecyltrimethylammonium bromide; Fipes, 1,4-piperazinediethanesulphonic acid.

Iron deficient Tris medium (Smith and Neilands, 1984). Used to elicit production of siderophores.

Trizma base	12.1g	
Glutamine	lg	
Succinic acid	1g	
KH ₂ PO ₄	0.1g	
K ₂ HPO ₄	0.1g	
MgS0 ₄ .7H ₂ 0	0.2g	
CaCl ₂ .2H ₂ 0	0.1g	
Na ₂ SO ₄	0.014g	
Pyridoxine. HCl	0.2mg	
Inositol	lmg	
Thiamine	1mg	
Pantothenate	lmg	
Biotin	lmg	
H ₂ 0	1 litre	
pH	6.8	

After autoclaving, casamino acids were added to a concentration of 0.3% w/w.

Casamino acids (Difco Lab.) for this medium and the CAS medium

were deferrated according to the method described byWaring 1942. The casamino acids were dissolved in 200ml of ultra pure water and filtered into a glass-stoppered separatory funnel. 8-OH quinolone (5mg) was dissolved in 1ml of chloroform, poured into the funnel, shaken vigourously and allowed to stand for five minutes. Approximately 3ml of chloroform were poured into the funnel, shaken vigourously for one minute and then rotated for 30 seconds to cause droplets of chloroform containing 8-OH quinolone-iron complexes to coalesce and settle. The chloroform layer was drawn off and the casamino acid solution washed twice with 3ml aliquots of chloroform. The whole extraction was repeated 3 times. The procedure removed all the contaminating iron.

Ethylenediamine-di-o-hydroxyphenyl acetic acid (EDDA), used in the above medium to chelate excess iron, was also freed from contaminating iron according to the method of Rogers (1973). A solution containing 10g of EDDA in 100ml of boiling IN HCl was prepared and allowed to cool. The solution was then filtered, diluted with 1.5 litres of acetone and the pH raised to 6 by the addition of 1N NaOH. After standing overnight at 4°C, the precipitate was filtered off and washed with cold acetone. The yield was approximately 75%.

YM medium.

Used for long term storage of Rhizobium strains.

K ₂ HPO ₃	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	0.1g
Yeast Extract	0.4g
Mannitol	10g
H ₂ 0	1 litre
pH	6.9

M9 medium (Maniatis *et al* ., 1982) Used for selection of *E.coli*. Na₂HPO₄ 6g

KH PO	3g				
NaCl	0.1g				
NH ₄ Cl	1g				
pH	7.4				
After autoclaving:					
1M MgSO4	2m1				
20% Glucose	10 m 1				
1M CaCl	0.lml				
were added as sterile	solutions to a	total	volume	of l	litre.

LB MgMal

Used for growth of E.coli prior to infection with Lambda.

Tryptone	10g
Yeast Extract	5g
NaC1	5g
Glucose	lg
Maltose	4g
MgCl ₂	lg
H ₂ O	1 litre.

Plant Test medium (Jensen, 1942) Used for growth of plant seedlings. Solution 1:

	MgSO4	0.2g
	K HPO	0.2g
	CaHPO	lg
	NaCl	0.2g
	FeC1	0.1g
	HO	1 litre
Solution	2:	
	CuSO ₄ .5H ₂ O	0.35g
	MnSO . 4H 0	6g
	ZnSO .7H 0	0.97g
	HBO	 12.7g
	NaMoO . 2H O	3.98g
	но	100m1
	6	

Solutions 1 and 2 were autoclaved separately and 250μ l of solution 2 were added to 1 litre of solution 1. Plates were allowed to set at an angle.

2.4 Surface sterilisation of seeds used in plant tests.

Seeds were washed in concentrated sulphuric acid for 10-15 minutes in a sterile flask, rinsed well with sterile water and washed for a further 10-15 minutes with commercial bleach. Seeds were then rinsed six times with sterile water and left standing in water for at least 4 hours. They were then placed on TY agar, (2.3), incubated upside down, and left to germinate in the dark at room temperature.

2.5 Sterilisation of nodules.

Nodules were carefully removed from the roots of clover plants and placed in a petri dish containing 70% ethanol for approximately 2 minutes. The nodules were then rinsed by transferring them aseptically from the ethanol into a petri dish of sterile water. This was repeated six times. Individual nodules were then placed on a petri dish in a drop of sterile water, crushed with a sterile spatula and a loopful of the exudate streaked onto a TY agar (2.3) plate.

2.6 Buffers and Solutions.

Chrome Azurol S solution.

All solutions and dilutions were made with 1mM Pipes, pH 5.6, and not with water as outlined in the procedure described by Schwyn and Neilands, 1987, unless otherwise stated.

A 6ml volume of 10mM HDTMA solution was placed in a 100ml volumetric flask and diluted. A mixture of 1.5ml iron (III) solution (1mM FeCl₃, 10mM HCl) and 7.5ml 2mM CAS solution was slowly added under stirring. A 4.307g quantity of anhydrous piperazine was dissolved and 6.25ml of 12M hydrochloric acid was carefully added. This buffer solution was rinsed into the

volumetric flask which was then filled with the pipes solution to give 100ml of CAS assay solution.

Hathway's Reagent,

One volume of iron reagent (0.1M FeCl₃ in 0.1M HCl) was added to one volume of 0.1M potassium ferricyanide.

TE Buffer

Tris-HCl	LOmM
Na ₂ -EDTA	1mM
pH	8.0

TES Buffer

Tris-HCl	1.0mM
Na - EDTA	1mM
NaCl	50mM
рН	8.0

Tris Borate Buffer (10X)

Tris-HCl	108g
Na ₂ -EDTA	9.3g
Boric acid	55g
H O	1 litre
pH	8.3

Solutions for mini-preps of plasmid DNA: Solution 1

Glucose 0.5M	1m1
EDTA 0.1M	lml
Tris-HCl 1M	0.25m1
но	7.75ml

Solution 2 (made every month and stored at room temperature) NaOH 1N 2ml SDS 10% 1ml H₂O 7ml Solution 3

Potassium acetate 3M pH 4.8

(To 60ml of 5M Potassium acetate, 11.5ml of glacial acetic acid and 2.85ml of H_2^0 were added. The resulting solution is 3M with respect to potassium and 5M with respect to acetate.)

Kirby Mix

	Phenol	100g
	Chloroform	100m1
	Isoamyl alcohol	4m1
	8-0H quinolone	0.8g
Solution	was stored under	100mM Tris-HCl, pH 7.5 at 4°C.

Solution for Maxi-preps of plasmid DNA:Triton Mix Triton X 100 20% 5ml EDTA pH 8, 0.25M 12.5ml Tris-HCl 1M 2.5ml H₂O to 50ml

Solutions for Gene-Clean Procedure: Sodium Iodide

> NaI 90.8g H₂O to 100ml

The solution was stirred until as much as possible was dissolved and then filtered through Whatman No.1 filter paper. 1.5g of Na SO_{2 3} were added and the solution stored at 4° C in the dark.

New Wash

	Ethano 1			50%	
	Tris	-HCl,	pH 7.	5	10mM
	EDTA				1mM
The	solution	was st	tored	at	- 20°C

Buffers for DNA digestion and ligation:

Restriction buffers were supplied by Bethseda Research Laboratories LTD.

Ligation Buffer (10X)

Tris-HCl	0.2M
MgCl ₂	0.1M
DTT	0.1M
ATP pH 7.6	0.06M

The buffer was stored at -20°C. Solutions for Southern Hybridisation: Depurination solution HCl 0.25M

Denaturing solution

NaCl	87.66g		
NaOH	20g		
H ₂ O to	l litre		

Neutralising solution

NaCl	87.6	6g
Tris-HCl pl	H 8,1M 500m	1
H ₂ O to	1 li	tre

Transfer	Buffer (20X SSC)	
	NaCl	175.83g
	Trisodium citrate	88.2g
	pH	7.0
	H ₂ 0 to	1 litre

Denhardt's solution

Ficoll	5g
Polyvinyl-	
pyrrolidone	5g
BSA(Pentax	
Fraction V)	5g

τ.

H₂O to 500ml

Prehybridisation solution

6 x SSC 5 x Denhardt's solution 0.5% SDS

100 μ g/ml denatured, fragmented salmon sperm DNA

Hybridisation solution

This solution is identical to the prehybridisation solution but contains in addition, EDTA (0.01M) and the 32 P-labeled DNA.

Solutions for *in situ* hybridisation of bacterial colonies: Transfer buffer (20 x SSPE)

NaCl	3.6M
NaH PO (pH 7.4)	200mM
EDTA (pH 7.4)	20mM

Prewashing solution

Tris-HCl	(pH	8)	50mM
NaCl			lM
EDTA			lmM
SDS			0.1%

Prehybridisation solution

Formamide		50%	
Denhardt's	solution	5X	
SSPE		5X	
SDS		0.1%	
	1		1.0

Denatured salmon sperm DNA (100µg/ml).

Solutions for Western Blotting: Transfer buffer Tris-HCl 0.025M

Glycine	0.192M
Methanol	20%
The pH of the buffer ran	nged from 8.1 to 8.5 and was not
adjusted with acid or base.	
Solutions for Western Blot	Detection:
TBST (Tris-buffered saline	containing Tween)
Tris-HCl (pH 8)	lOmM
NaCl	150mM
Tween-20	0.05%

Blocking solution

TBST	
BSA	1% w/v

AP buffer (for alkaline phosphatase detection) Tris-HCl (pH 9.5) 100mM NaCl 100mM MgCl 5mM

Nitroblue tetrazolium solution (NBT) 50 mg/ml in 70% N,N-dimethylformamide.

5-bromo-4-chloro-3-indoyl phosphate solution (BCIP) 50 mg/ml in N,N-dimethylformamide.

AP colour developing solution

AP buffer	10m1
NBT	66 µ 1
BCIP	33µ1

The solution is light sensitive and must be used within one hour of preparation.

2.7 Antibiotics

Neomycin sulphate, strepomycin sulphate, ampicillin, kanamycin,tetracycline and rifampicin were from Sigma. Aqueous solutions of neomycin sulphate, streptomycin sulphate and kanamycin (20 mg/ml) were prepared and stored at 4° C. Tetracycline was dissolved in 50% ethanol and stored at 4° C. Ampicillin, dissolved in distilled water, and rifampicin, dissolved in methanol, were prepared as required.

2.8 Enzymes.

Restriction and ligation enzymes were from BRL and were used according to the manufacturer's instructions.

2.9 Storing and culturing bacteria.

Strains were stored as glycerol stocks. An equal volume of glycerol (80%) was added to lml of a late log phase culture and stored at -20° C. Where hosts were harbouring unstable plasmids, the appropriate antibiotic was added to the stock medium. Working stocks were stored on plates at 4° C.

2.10 Isolation of antibiotic resistant mutants.

A late log phase culture (10ml) was pelleted by centrifugation at 5000g for 10 minutes. The cells were resuspended in 1ml of fresh growth medium and 0.1ml aliquots plated on agar plates containing the appropriate antibiotic. Resistant colonies were purified on selective plates.

2.11 Bacterial conjugation.

Conjugations were performed on solid agar surfaces. *Rhizobium* recipients were grown to late log phase in TY broth while *E.coli* donors were grown to mid log phase in LB broth. Donor and recipient (0.7ml of each) were mixed in an eppendorf tube, centrifuged and resuspended in 100μ l of fresh TY broth. This suspension was placed on a 0.45μ m membrane filter on a TY agar plate and incubated overnight at 30° C. Filters were aseptically removed to 2ml of sterile water and the cells resuspended by vortexing. Dilutions (10^{-1} and 10^{-2}) of this suspension were plated on a selective medium. The donor and recipient strains were carried through the same procedure separately and plated on selective medium to check counter selection.

2.12 Bacterial transformation.

The *E.coli* strain routinely transformed was JA221. Competent cells were prepared by the following method: One ml of an overnight culture of JA221 was inoculated into 100ml of LB broth and allowed to grow to an 0.D.600 of 0.3-0.4. The culture was then chilled on ice for 30 minutes at which time a 10ml aliquot was pelleted by centrifugation at 6000rpm at 4°C for 5 minutes. The pellet was resuspended in 5ml of ice-cold MgCl₂ (0.1M). The cells were again centrifuged and resuspended in 5ml of ice the cells were centrifuged and resuspended in 1ml of CaCl₂ (50mM). After 30 minutes on ice the cells were now competent and were stored on ice for use. Plasmid DNA was transformed into the recipient strain by adding

200µl of the competent cells to 20μ l of DNA dissolved in TE buffer and allowing the mixture to stand on ice for 1 hour. The transformation mix was heat shocked at 42°C for 2 minutes and transferred immediately back to the ice. After addition of 0.8ml of fresh LB broth the mix was placed at 37°C for 1 hour to allow outgrowth of the cells. Cells were then plated on selective medium.

2.13 Preparation of plasmid DNA (rapid preparation).

The method used was that described by Birnboim and Doly (1979). The solutions used are described in section 2.6.

A bacterial culture (1.5ml) grown in selective medium was pelleted in a microcentrifuge and the supernatant removed. The pellet was resuspended by vortexing in $100\mu 1$ of solution 1 and left for 5 minutes at room temperature. Then $200\mu l$ of solution 2 were added, mixed by inversion and the tube placed on ice. After 5 minutes, 150μ l of solution 3 were added and the mixture kept on ice for 10 minutes. A clot of chromosal DNA had formed and was pelleted by centrifugation. The supernatant $(400\mu 1)$ was placed into а fresh tube and an equal volume of by vortexing. phenol/chloroform added and mixed After centrifugation the aqueous layer was removed to a new tube and an equal volume of isopropanol was added. After 10 minutes at

room temperature the tube was centrifuged for 5 minutes at 12,000 rpm. The pellet was washed twice with 70% ethanol, dried briefly in a dessicator and resuspended in 30μ l of TE buffer containing RNase (20μ g/ml).

2.14 Preparation of bulk small plasmid DNA.

Cells from a late log phase culture (250ml) were harvested by centrifugation and resuspended in sucrose (25% in 50mM Tris pH 8) to a volume of 2ml. The suspension was transferred to a plastic screw-capped ultra-centrifuge tube and 0.4ml of a lysozyme solution (20mg/ml in 0.25M Tris, pH 8) were added. After 5 minutes 0.8ml of EDTA (0.25M, pH 8) were added and after a further 10 minutes, 3.2ml of Triton lysis mix (see 2.4) were added. The tube was kept on ice during the above additions. After 15 minutes the suspension was centrifuged at 40,000 rpm at 4°C for 40 minutes. Caesium chloride (6.9g) was dissolved in the cleared lysate. This solution was transferred to a Quick Seal polyallomer ultracentrifuge tube (Beckman) and 0.18ml of an ethidium bromide solution (10mg/ml) were added. The total solution weight was brought to 14.1g using 10mM EDTA. Air was removed from the tube by the addition of liquid paraffin and the tube sealed with a Beckman heat sealer. The density gradient was formed by centrifugation at 50,000 rpm at 18°C in a Beckman Ultracentrifuge Model LH-M. After 20-22 hours the covalently closed circular plasmid DNA had separated from the linear chromosomal DNA. The DNA was visualised using an ultra-violet lamp and plasmid DNA was recovered by puncturing the tube with a syringe and withdrawing the plasmid band. Ethidium bromide was removed by extraction with an equal volume of isopropanol saturated with 20x SSC (2.6). The extraction was repeated approximately six times. To remove CsCl the DNA solution was transferred to dialysis tubing and dialysed against several changes of TE buffer.

2.15 Isolation of total DNA from Rhizobium strains.

Early stationary phase cultures of *Rhizobium* spp. (10ml) were sedimented in a Beckman J2-21 centrifuge at 10,000rpm for five

minutes. The cells were washed with an equal volume of TES buffer and resuspended in 5ml of TE buffer. 0.5ml of a freshly prepared lysozyme solution (2mg/ml in TE) was added and the suspension incubated at 30°C. After 20 minutes, 0.5ml of a sarkosyl/pronase solution (10% sarkosyl in TE containing 5mg/ml pronase) was added and the suspension incubated at 37°C for one hour. Lysis was evident by an increase in viscosity of the suspension. Sodium acetate (0.7ml of 3M) was added and mixed gently. Kirby mix (2.5ml) was added and the suspension was mixed slowly inversion for 15-30 minutes. After by centrifugation at 10,000rpm for ten minutes the aqueous phase was removed to a glass corex tube and centrifuged at 10,000rpm for fifteen minutes. The supernatant was removed and 2.5ml of kirby mix was added. The suspension was mixed gently for fifteen minutes before extraction with chloroform; isoamyl alcohol (24:1). The DNA was precipitated with an equal volume of isopropanol and was evident in the suspension as a coiled thread. The DNA was removed to a microcentrifuge tube using a pipette, centrifuged, washed twice with 70% ethanol, dried under vacuum and resuspended in 400μ l of TE buffer.

2.16. Agarose gel electrophoresis for DNA characterization.

A horizontal gel apparatus (Pharmacia) was used. All DNA samples were loaded on 0.8% agarose gels made in TBE buffer. Mini-gels were run at approximately 100V for one hour while maxi-gels were run at 50V overnight. A tracker dye was incorporated into DNA samples to facilitate loading. Gels were run in TBE buffer (1 x) until the tracker dye had reached the base of the gel. DNA was visualised by staining for thirty minutes in ethidium bromide, then placing the gel on a U.V. transilluminator. Gels were photographed using a polaroid land camera equipped with a red filter (25A Kodak) loaded with polaroid 667 positive film.

2.17. Isolation of DNA from agarose gels:-gene clean

procedure.

After electrophoresis and staining, the DNA band of interest was cut from the gel and weighed. Two to three volumes of a saturated sodium iodide solution (2.6) was added to the agarose band and incubated at 55°C for five minutes or until all the agarose had dissolved. Silica 325 mesh glass beads (5μ) obtained from Stratech Scientific Ltd. were added to the solution, mixed and allowed to stand at room temperature for five minutes. The glass beads were centrifuged in amicrofuge for five seconds at 12,000rpm. The pellet was washed three times with 10-50 volumes of ice-cold NEW wash (2.6), spinning for five seconds between each wash. The DNA was eluted from the pelleted beads by resuspending in a small volume (10-20 μ 1) of TE and incubating for three minutes at 55°C. The supernatant containing the DNA was stored at 4°C.

2.18. Southern blotting - transfer of DNA to nitrocellulose filters.

The technique used was that described by Southern (1975). After electrophoresis was completed and the DNA stained with ethidium bromide, the gel was photographed. The gel was transferred to a tray containing several volumes of denaturing solution and incubated on a shaking table at room temperature for 45 minutes. The denaturing solution (2.6) was poured off and the gel was soaked for one hour in neutralising solution with constant shaking. At this point the pH of the surface of the gel was below 8.5. A piece of Whatman 3MM paper longer and wider than the gel was soaked in 20 x SSC, placed on a support and connected to a resevoir of 20 x SSC. The gel was placed on the Whatman paper and a piece of nitrocellulose (Schleicher and Schnell) presoaked in 20 x SSC and exactly the size of the gel was placed on top. Three pieces of Whatman 3MM paper cut to exactly the same size as the gel and soaked in 20 x SSC were placed on top of the nitrocellulose filter. A stack of paper towels (5-8cm high) just smaller than the 3MM paper were placed on top. A glass plate was put on top of the towels and weighted down with a 500g weight. To prevent short-circuiting of fluid

between the towels and the 3MM paper under the gel it was surrounded by a water-tight border of parafilm. Transfer was allowed to procede for 24 hours. The nitrocellulose filter was then removed, soaked in 6 x SSC at room temperature for five minutes, air dried, then baked for two hours at 80° C.

2.19. Preparation of radioactive probe.

Nick translation: (Rigby *et al.*, 1977). The reaction consisted of: 10 x Nick translation buffer DNA probe $(0.1\mu g/\mu l)$ 10 μl dGTP (10mM) 1 μl dCTP (10mM) 1 μl dTTP (10mM) 1 μl [α -³²P] dATP (800 Ci/mM) 1 μl H₀ to μl

The mixture was chilled to $0^{\circ}C$ and $0.5\mu l$ of diluted DNase 1 $(0.1\mu g/m l)$ was added. Five units $(1\mu l)$ of DNA polymerase 1 was added and the reaction incubated at $16^{\circ}C$ for one hour. The reaction was stopped by adding $2\mu l$ of 0.5M EDTA and the nick-translated DNA was separated from unincorporated dNTPs by gel filtration through a Sephadex G-50 column (Maniatis *et al.*, 1982).

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Random Priming:
```

The Prime-a-Gene (Promega) labeling system based on the method developed by Feinburg and Vogelstein (1983) was used.

The reaction consisted of:

5 x labeling buffer	10 <i>µ</i> 1
Mixture of 3 unlabeled dNTPs	$20\mu M$ each
Denatured linear DNA template	500ng/ml
Acetylated B.S.A. (1mg/m1)	2μ l
$[\alpha^{-32}P]$ dNTP (3000 Ci/mM)	5µ1
Klenow enzyme	5U
Sterile water to	50µ1
The components were mixed	gently and the

The components were mixed gently and the reaction tube incubated at room temperature for sixty minutes. The reaction

was terminated by heating at $95^{\circ}-100^{\circ}$ C for two minutes and subsequently chilling in an ice bath. EDTA was added to 20mM and the mixture was used directly in a hybridization or stored at -20° C for later use.

2.20. Hybridization of Southern filters.

The baked filter was soaked in 6 x SSC for two minutes and slipped into a heat-sealable bag. Prehybridization fluid $(0.2ml/cm^2$ of nitrocellulose filter) warmed to $68^{\circ}C$ was added to the bag. After squeezing air from the bag it was sealed and incubated at 68° C on a shaking table for two to four hours. After prehybridization the fluid in the bag was replaced with hybridization solution $(50\mu l/cm^2$ of filter) containing the labeled denatured probe. Hybridization was allowed to continue for 16-20 hours at 68°C. After hybridization, the fluid was removed from the bag and the filter was transferred to a tray containing a solution of 2 x SSC and 0.5% SDS and incubated at room temperature for 15 minutes. This solution was then replaced with a solution of 2.0 x SSC and 0.1% SDS and the filter incubated for a further 15 minutes. Finally, the filter was incubated for 2 hours at 68°C in a solution of 0.1 x SSC and 0.5% SDS. The buffer was then changed and incubation continued for another 30 minutes. The filter was air dried at room temperature on a sheet of Whatman 3MM paper, placed in a plastic heat sealable bag and applied to kodak x-ray film. After exposure, the film was developed and fixed using kodak DX-80 developer and kodak FX-40 x-ray liquid fixer.

2.21. In situ hybridization of bacterial colonies.

Nitrocellulose filters were placed on agar plates containing the selective antibiotic. Individual bacterial colonies were transferred (using sterile toothpicks) onto the filters and then onto a master agar plate. The plates were incubated at 37° C until colonies had reached 0.5-1.0mm in width. The nitrocellulose filters were placed colony side up on 3MM Whatman paper impregnated with the following solutions:

10% SDS (3 minutes), denaturing solution (5 minutes),
neutralising solution (5 minutes) and 2 x SSPE (30 minutes). The filters were air dried, colony side up, for 30-60 minutes and were then baked on a sheet of 3MM paper at 80° C for 2 hours.

2.22. Hybridization to nitrocellulose filters containing replicas of bacterial colonies.

The baked filters were floated on the surface of a 6 x SSC solution, then immersed for 5 minutes. The filters were then stacked on top of one another in a glass petri dish. Prewashing solution (20ml) was added and the filters incubated at 42°C for 2 hours on a shaking table. The prewashing solution was poured off and the filters were transferred to a heat sealable plastic and incubated for 4-6 hours at 42°C in bag 12m1 of α^{32} P-labeled probe DNA was prehybridization solution. The denatured by heating for 5 minutes to 100°C and it was added to the prehybridization solution. Hybridization continued for 16 hours. After hybridization the solution was discarded and the filters washed 3-4 times, for 5-10 minutes each wash, in a large volume of 2 x SSC and 0.1% SDS at room temperature. The filters were taken washed twice for 1 hour in 100ml of a solution of 1 x SSC and 0.1% SDS at 68° C. The filters were air dried, placed in a heat sealable plastic bag and exposed to x-ray film as described for Southern transfer filters.

2.23. Preparation of outer membranes for SDS-PAGE.

One ml of a stationary phase culture was added to 250μ l of Tris medium containing various contrations of iron. Cells were harvested by centrifugation at 50,000 rpm for 5 minutes, washed with 10mM tris pH 7.5, pelleted and the wet weight determined. Cells were sonicated (15 bursts of 20 seconds with 40 second rests) in an ice-ethanol bath. After sonication, DNase and RNase to a concentration of 10μ g/ml were added and were placed at 22°C for 1 hour. Cell debris and unlysed cells were pelleted by centrifugation at 10,000 rpm for 15 minutes. Supernatants were decanted into screw-capped ultracentrifuge tubes and cell envelopes were pelleted by spinning at 50,000 rpm for 30 minutes. Outer membranes were prepared by treating the cell envelope preparation with 1% sarkosyl which selectively dissolves the inner membrane. This procedure was repeated once. Outer membrane preparations were solubilised in a buffer containing 25mM Tris, pH 6.8, 0.5% mercaptoethanol, 1% SDS, 1mM EDTA, 50% glycerol and 0.005% bromophenol blue.

2.24. SDS polyacrylamide gel electrophoresis.

Samples were routinely run on 10% SDS polyacrylamide gels.Gels were prepared as follows:Separating gel 10%Bisacrylamide solution10ml1M tris pH 8.811.2mlH_08.7ml10% SDS0.3mlTemed0.02mlAnmonium persulphate (10%)0.1ml

Stacking gel	
Bisacrylamide solution	1m1
1M tris pH 6.8	1.25m1
H ₂ O	7.7ml
SDS (10%)	0.1ml
Temed	0.02m1
Ammonium persulphate (10%)	0.075ml
The bisacrylamide solution contained	:
Bisacrylamide	1.6g
Acrylamide	60g
H_O	200ml

The solution was stored in the dark at $4^{\circ}C$. Gels were routinely run at 30mA for approximately 6-7 hours. Running buffer contained 6.055g tris, 28.82g glycine and 2g SDS/21. After electrophoresis gels were stained with Coomassie Blue, destained and stored in 7% acetic acid.

Destain solution contained:

Methanol				400ml			
H ₂ 0				500ml			
Acetic acid				50m1			
Coomassie blue was	added	to	the	destain	solution	(5mg/l)	to
prepare the stain.							

2.25. Preparation of gel fragments prior to use as antigens. Proteins to be used as antigens were cut out of SDS-PAGE gels, minced finely and suspended in 0.5ml 1 x PBS containing 0.1% triton-X. Proteins were allowed to diffuse out of the gel fragments overnight at 37°C. This suspension was mixed with 0.5ml of Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for subsequent injections. Prior to injection the mixture was emulsified by sonication.

2.26. Western blotting.

Following electrophoresis, the SDS polyaceylamide gel was equilibrated in transfer buffer for 45 minutes. A piece of nitrocellulose filter was cut to the dimensions of the gel and allowed to soak in transfer buffer for 15-30 minutes. Two pieces of filter paper per gel were also saturated in transfer buffer. A Trans-Blot Electrophoretic Transfer cell apparatus (Biorad) was used in the Western Blot procedure. A gel/membrane sandwich was constructed according to the diagram below:

filte

nitrocellulose membrane

The gel/membrane sandwich was placed between the two pre-wetted fibre pads of the transfer apparatus, taking care that the gel was closest to the cathode panel. Transfer proceeded at 60V for 5 hours. Following transfer, the membranes were stored wet at 4°C in heat-sealed plastic bags or used immediately.

Procedure for Western Blot detection of proteins. 2.27.

All washing and incubation steps were carried out at room temperature with gentle agitation.

The nitrocellulose membrane was floated on TBST until evenly

wet. To saturate non-specific protein binding sites, the TBST was replaced with blocking solution. After 30 minutes this solution was decanted and replaced with TBST containing the desired dilution of primary antibody. The Western blot membrane was incubated with the anitbody for 1-2 hours. To remove unbound antibody, the membrane was washed three times with TBST for 5-10 minutes each. The membrane was then transferred to TBST containing the anti-1gG alkaline phosphatase conjugate and incubated for 30 minutes. After washing the membrane three times with TBST for 5-10 minutes each, the alkaline phosphatase colour reaction was carried out. The membrane was blotted dry on filter paper and transferred to 10ml of the alkaline phosphatase development solution. Reactive areas turned purple, usually within 1-15 minutes. The reaction was stopped by rinsing the membrane in deionized water for several minutes.

Chapter Three

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Detection, isolation and partial characterisation of a siderophore from *Rhizobium meliloti* 220-5.

3.1 Introduction

The majority of siderophore molecules fall into either the hydroxamate or catechol classes. Both functional groups are chemically detectable by colourimetric assays: catechols via Arnow's method (Arnow, 1937) and hydroxamates via the Csaky test (Csaky, 1948). Recently, mugineic acid, a phytosiderophore and rhizobactin, a structurally novel type of siderophore, have been the focus of interest. Using amine, carboxylate, and alcoholate groups as binding sites for iron(iii), such chelates lack specific moieties for chemical assay and their complexes are only weakly coloured in the visible region of the spectrum. Until recently, to detect siderophores in such cases, only biological assays, which are sensitive but tedious, were possible. In 1987, Schwyn and Neilands reported on the development of a Universal Chemical Assay for the detection of siderophores. The assay which is based on the affinity of the siderophores for iron is independent of their structure. The following equation explains the principle:

$\operatorname{FeDye}^{3-\lambda} + L^{\kappa} \longrightarrow \operatorname{FeL}^{3-\lambda} + \operatorname{Dye}^{\lambda-1}$

A strong ligand L, a siderophore, is added to a highly coloured iron dye complex. When the complex between the iron and the ligand is formed, the release of free dye is accompanied by a colour change. The dye, composed of a ternary complex of Chrome Azurol S, hexadecyltrimethylammonium bromide (HDTMA) and Fe^{3+} , is blue but turns to orange once the Fe^{3+} moiety is removed. The dye can be used in either a plate or solution assay. 3.2 Siderophore production is strain specific in *Rhizobium* species.

Chrome Azurol S blue agar plates were prepared as already described (2.3) and siderophore production in a variety of Rhizobacteria was tested by growing strains on the CAS plates and observing a colour change from blue to orange indicating the presence of a siderophore. Table 3.1 summarizes the results observed.

Table 3.1

		Production of	siderophore
Rhizobium meliloti	2011	+	
	DM4	+	
	220-5	+	
	220-3	+	
	220-24	+	
	220-1 to -24		
(excludi:	ng the above)		
	102F34		
	65		
Rhizobium legumino	sarum	<i>V</i>	
biovar viciae	VF39	+	
	JI 300	+	
	B16 4	-	
	3855		
	B151	1.2	

Rhizobium legumin	osarum	
biovar trifolii	1027	+
	FYM2	+
	FYM1	0.40
	RCR 5	

	32	÷
	46	÷
	221	
Rhizobium loti	LP22	-
	LP27	÷.
	LP28	-
	N2P2042	-

Agrobacterium tumefaciens

1060	Ri plasmid	1.5
C58		1÷
9023	Plasmid free	
4011		-

.

Pseudomonas fluorescens DN12 Results from the CAS assay indicate that siderophore production is strain specific in Rhizobium species. This strain specificity was also observed when R.trifolii strains were isolated from nodules present on clover plants grown in high pH soils. Alkaline soils were chosen because at pH 7 or greater, iron is virtually insoluble (Biedermann et al., 1957) and Rhizobium strains growing in such an environment would be expected to have a higher incidence of siderophore production compared with Rhizobium strains in a more acid environment, such as the bradyrhizobia from tropical soils (Guerinot et al., 1990), where iron is more readily available. Four alkaline soils were chosen and planted with white clover seeds (varieties Huia and Blanco) which had been surface sterilised and germinated (2.4) for 2-3 days at room temperature on TY plates. After four weeks nodules were removed from plant roots, sterilised and the Rhizobium strains isolated

(2.5). Once purified these strains were tested for siderophore production on CAS agar plates. The results of this analysis are presented in Table 3.2.

Table 3.2

Soil	pН	% Total	No.Isolates	No.+ve	<pre>% Positive for</pre>
		Iron	Tested		Siderophore.
1	7.6	1.04	40	17	40
2	8.6	0.67	84	48	54
3	7.5	0.13	63	25	40
4	7.5	2.20	32	5	16

3.3 Siderophore production by Rhizobium meliloti 220-5.

To date the most detailed work regarding the production of siderophores by *Rhizobium* species has been carried out using *Rhizobium meliloti* strains (Smith and Neilands, 1984, 1985; Gill and Neilands, 1989). The work presented for this thesis is concerned with the production of a siderophore by *Rhizobium meliloti* 220-5 which gives a positive reaction for siderophore in the CAS assay.

3.3.1. The effect of iron on the growth of *R.meliloti* 220-5. Aliquots (lml) of an overnight culture of *R.meliloti* 220-5 grown in TY broth were inoculated into four 250ml flasks of low iron Tris medium (2.3) containing different iron concentrations. Flasks were incubated at 30° C at 150rpm and growth was allowed to proceed for 24 hours. Samples were taken from each of the cultures at three hourly intervals and the optical density at 600nm measured. The resulting growth curves are presented in Fig. 3.1.

From the graph it can be seen that, when the iron concentration is strictly limiting i.e. when the iron chelator EDDA is added to the medium, the growth of *R.meliloti* 220-5 is severely retarded. When 220-5 is grown in the Tris medium only, which contains $<1\mu$ M Fe, its growth is also limited but not as severely compared with its growth at higher iron concentrations.

Growth of R. meliloti 220–5 in different iron concentrations



Fig. 3.1

3.3.2 Production of siderophore by R.meliloti 220-5.

Using the Chrome Azurol S solution assay (2. 6), samples taken from the culture grown in Tris medium were tested for the siderophore. A 0.5ml aliquot of cell-free presence of supernatant from each sample was mixed with 0.5ml CAS assay solution. A reference was prepared using exactly the same components except the siderophore (e.g. the uninoculated medium used to culture R.meliloti 220-5). After reaching equilibrium the absorbance was measured at 630nm. Fig. 3.2 shows the qualitative results of the assay i.e. the check for the presence of siderophore by watching the colour change from blue to orange. Fig. 3.3 gives the results of the absorbance readings measured at 630nm. For the Fe-CAS-HDTMA complex an extinction coefficient of approximately 100,000 M⁻¹cm⁻¹ was found at 630nm in solutions buffered at pH 5.6 (Schwyn and Neilands, 1987). At this wavelength the orange-coloured, iron-free complex has essentially no absorbance. The main application of the CAS assay solution is the qualitative check for the presence of siderophores in culture supernatants. From Fig. 3.2 it is observed that the production of siderophore commences between 12 and 15 hours into the growth cycle. This corresponds to the onset of exponential growth seen in Fig. 3.1.



Hours

Fig 3.2

Production of siderophore by *R.meliloti* 220-5 over a 24 hour growth period.



Production of Siderophore during the growth cycle of R.meliloti 220-5.

Fig. 3.3

3.3.3. Classification of the *R.meliloti* 220-5 siderophore. As most siderophores belong to either the cathecolate or hydroxamate classes, the cell-free culture supernatant of R.meliloti 220-5, shown to contain a siderophore, was tested for the presence of these groups. All assays were used as qualitative rather than quantitative tests for their presence. The Arnow assay (Arnow, 1939) is the assay most commonly used to detect the presence of cathecol groups. The assay was originally developed to determine levels of 3,4-dihydroxyphenylalanine and is based on the fact that this substance gives a yellow colour with nitrous acid, the yellow colour changing to an intense orange-red in the presence of excess sodium hydroxide. The same reaction occurs with cathecols. The assay procedure is as follows: One ml of the test solution was pipetted into a test tube. To this was added, in the order given, 1ml of 0.5N hydrochloric acid and 1ml of 1N sodium hydroxide and 2ml of water mixing well after each addition. The results of the Arnow assay are given in Table 3.3 and Fig. 3.4. 4

Colo	ur after HCl and	Colour after
Sample	Molybdate	NaOH
Distilled H_2^0	Colourless	Colourless
2,3 DHBA [*]	Yellow	Red
3,4 DHBA [*]	Yellow	Red
Anthranilate	Colourless	Colourless
R.meliloti supernatant (24hrs)	Colourless	Colourless
<i>R.meliloti</i> supernatant (60hrs)	Faint yellow	Colourless
Tris medium	Colourless	Colourless

* DHBA = Dihydroxybenzoic acid.



Fig 3.4

Tube	1:	H ₂ O			
Tube	2:	2, 3 - DHBA			
Tube	3:	3,4-DHBA			
Tube	4:	Anthranilate	2		
Tube	5:	Supernatant	(60	hours)	
Tube	6:	Supernatant	(24	hours)	

The results indicate that the siderophore produced by R.meliloti 220-5 is not a catechol.

A more sensitive method for the determination of catechols was developed by Rioux in 1983. The production of catechol was detected in supernatant fluids of a *R.leguminosarum* strain where a negative result had been obtained with the Arnow assay. The assay is based on the reduction, in acidic conditions, of Fe^{3+} to Fe^{2+} by the vicinal hydroxyl groups on the catechol. The following reagents were pipetted into a polystyrene tube in the order given:

2.3ml H₂O 0.2ml H₂SO₄ (20% v/v)

to provide acidic conditions.

1.Oml	Test sample	
0.1m1	Ferric ammonium citrate	to provide the Fe $^{3+}$
	(1% w/v)	substrate
0.4ml	Ammonium fluoride (2M)	to mask excess ${\rm Fe}^{3^+}$
0.4ml	1,10 phenanthroline (1%w/v)	reacts with Fe $^{2+}$
0.6ml	Hexamethylenetetramine (3M)	provides a buffer for the
	1	above reaction.

The assay mixture was vortexed after each addition and heated for one hour at 60° C after the final addition. To correct for any contamination of the assay mixture by Fe²⁺ a blank was prepared whereby 1ml of distilled water replaced the sample. Absorbance was measured at 510nm and results are presented in Table 3.4.

Table 3.4.

	C	orrected Abs.	Corrected Abs
Sample	Abs	for blank	for Tris medium
Blank	0.081		
2,3 DHBA $2\mu M$	0.468	0.367	
$4\mu M$	0.808	0.727	
6μ M	1.068	0.987	
8μ M	1.486	1.405	
Supernatant			
R.meliloti 220-5	0.157	0.076	0.020
Supernatant			
R.meliloti 2011	0.232	0.151	0.045
Supernatant			
R.meliloti 102F34	0.180	0.099	0.043
Supernatant			
P.fluorescens DN12	0.307	0.226	0.170
Supernatant			
A.tumefaciens 1060	0.670	0.589	0.533
4μM Hydroxylamine	0.092	0.011	0.011
80µM Hydroxylamine	0.088	0.007	0.007
Tris medium	0.137	0.056	

The results of the Clement Rioux assay also indicate that the siderophore in the supernatant of *Rhizobium meliloti* 220-5 is not a catechol.

To assay for the hydroxamate group, three methods described by Gillam *et al.*, 1981 were used. Csaky Test 1948 This test for bound hydroxalamine involves the hydrolysis of the sample with 3M sulphuric acid at 130°C for 1 hour. The procedure is as follows: An aliquot (2ml) of the test sample was pipetted into a

test-tube. Sulphuric acid (2ml, 3M) was added and the solution hydrolysed. After cooling, the hydrolysed solution was rinsed into a 50ml volumetric flask and the following solutions added:

7ml 2M sodium acetate

2ml Sulphanilamide solution (1% w/v in 30% v/v acetic acid)

2ml Iodine solution (0.65% w/v in 1% w/v KI solution)The mixture was swirled and allowed to react for 5 minutes. Then the following were added:

2ml Sodium arsenite (1.5% w/v in distilled water)

2ml N-(1-Naphthyl) ethylenediamine solution

(0.05% w/v in distilled water)

The reaction mixture was left for 30 minutes for complete colour development, at which time it was diluted to 50ml and the absorbance measured at 543nm. A blank determination was made by addition of the reagents to an aliquot (2ml) of acid hydrolysed distilled water. The results are presented in Table 3.5.

Table 3.5.

Sample	Absorbance @ 543nm
Hydroxylamine (1mg/ml)	0.748
Acetohydroxamic acid (lmg/ml)	0.759
Supernatant of	,
R.meliloti 220-5	0.011
Tris medium	0.000
2,3 DHBA (1mg/ml)	0.053

Periodic Acid Test

This test involves the oxidation of certain hydroxamic acids with periodic acid. The oxidation of free or acylated N-alkyl-hydroxylamines produces the corresponding cis-nitroso dimer whose $\lambda \max = 264 nm$.

An aliquot (1ml) of the test sample was pipetted into a 10ml volumetric flask. Periodic acid (2.5ml of a 50mg/100ml stock), 2ml of distilled water and 5 drops of glycerol were added. The flask was shaken to mix the solution, the volume was made up to 10ml with distilled water and the absorbance of the final solution measured at 264nm. A blank was prepared using distilled water as the sample. The results are presented in Table 3.6.

Table 3.6.

Sample	Absorbance @	264nm
Hydroxylamine (lmg/ml)	0.062	
Acetohydroxamic acid (lmg/ml)	0.013	
Desferal (lmg/ml)	1.555	
Supernatant	I	
R.meliloti 220-5	0.040	
Tris medium	0.020	

Primary hydroxamic acids and hydroxylamine do not produce the nitroso dimer thus accounting for the low readings observed for hydroxylamine and acetohydroxamic acid in the above assay.

Berg and Becker Test

This test involves the direct determination of hydroxylamine by the dimerization of 2 mol of 8-hydroxyquinolone with 1 mol of hydroxlamine.

An aliquot (2ml) of the test sample was pipetted into a 50ml volumetric flask and hydrolysed with an equal volume of 3M sulphuric acid for one hour at 130° C. After hydrolysis, 14ml of a 1M solution of NaOH and 2ml of a 1M solution of Na₂CO₃ were added to the hydrolysis mixture to adjust the pH to the value required for the remainder of the analysis (pH 11.0 ⁺ 0.5). 8-hydroxyquinolone (2ml, 1% w/v in 100% ethanol) was added, the

solution shaken and left for one hour for complete colour development. A blank was prepared using distilled water as the test sample. The absorbance of the final solution was measured at 700nm. The development of a green indooxine complex indicated the presence of an hydroxamate group. Results of the assay are given in Table 3.7 and Fig. 3.5.

Table 3.7

Presence of indooxine	Absorbance		
complex	@ 700nm		
+	1.827		
	0.034		
+	0.345		
	0.000		
	0.000		
	0.000		
	Presence of indooxine complex + + - + - -		



Fig. 3.5

TUBE	1	Hydroxylamine				
	2	Desferal (unhydrolysed)				
	3	Desferal (hydrolysed)				
	4	R.meliloti 220-5 supernatant (unhydrolysed)				
	5	R.meliloti 220-5 supernatant (hydrolysed)				
	6	Low iron Tris medium (unhydrolysed)				
	7	Low iron Tris medium (hydrolysed)				

The results of the three assays for the determination of the hydroxamic acid group in unknown siderophores indicate that the siderophore of R.meliloti 220-5 is not a hydroxamate.

A report by Guerinot *et al.*, 1990 demonstrated that citric acid is produced and used by certain *Bradyrhizobium* strains to sequester iron. To test the possibility that the 220-5 siderophore being produced could be citric acid, the low-iron culture supernatant was assayed for citric acid.

The Boehringer-Mannheim assay kit for citric acid was used. The following reactions form the basis for the assay:

(i) Citrate Citrate lyase Oxaloacetate + Acetate

In the presence of the enzymes malate dehydrogenase (MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, by reduced nicotinamide-adenine dinucleotide (NADH).

(ii) Oxaloacetate + NADH + $H^+ \xrightarrow{MDH}$ L-malate + NAD⁺ (iii) Pyruvate + NADH + $H^+ \xrightarrow{L-LDH}$ L-lactate + NAD⁺

The amount of NADH oxidised in reactions (ii) and (iii) is stoichiometric with the amount of citrate. NADH was determined by its absorbance at 340nm. An initial absorbance Al determined the amount of NADH present before the above reactions took place; a second absorbance A2 determined how much NADH had been oxidised in the reactions. Results of the assays are give in Table 3.8. Table 3.8.

Sample	A1	A2	ΔA			
Blank	1.436	1.415	0.021			
Standard Citric						
Acid Solution (0.397g/L)	1.417	0.560	0.836			
Supernatant						
R.meliloti 220-5 (24hrs)	1.419	1.418	0.001			
Supernatant						
R.meliloti 220-5 (60hrs)	1.417	1.416	0.001			

Using the ΔA_{340} of 0.836 obtained in the assay, the concentration of the standard citric acid solution (0.397g/L) was determined to be 0.385g/L, indicating the accuracy of the assay. No change in the NADH concentration after the addition of citrate lyase was observed when the supernatant containing the *R.meliloti* 220-5 siderophore was tested indicating that the siderophore is not citric acid.

The results of the assays to detect catechol and hydroxamate siderophores suggest that the siderophore of *R.meliloti* 220-5 belongs to the "rhizobactin" class of siderophores and following the example of Gill and Neilands (1989) where the siderophore from *R.meliloti* 1021 was named rhizobactin 1021, this siderophore has been named rhizobactin 220-5. 3.4. Iron-nutrition bioassays to detect siderophore activity

EDDA (2.3) chelates iron (III) with an apparent stability constant near 10^{30} . Bacterial growth inhibition by EDDA-induced iron depletion is alleviated by specific iron transport agents or by excess iron. To carry out the iron nutrition bioassays, 200μ l of an overnight culture of *Rhizobium* was inoculated into 25ml aliquots of molten Tris medium and poured into sterile plates.Wells, 50mm in diameter, were cut out of the agar plates and 20μ l of the test solution pipetted into the wells. Growth was allowed to proceed for 24-48 hours at 30° C and plates were examined for halos of bacterial growth surrounding wells bearing bioactive test solutions.

Assays were carried out on *R.meliloti* DM4, *R.meliloti* 65, *R.meliloti* 102F34 and a siderophore negative mutant of *R.meliloti* 2011 and 220-5. The results of the bioassays (Table 3.9) gives further evidence for the strain specificity of siderophore production in *Rhizobium* species since it was shown that *R.meliloti* DM4, 65 and 102F34 cannot use the siderophore produced by *R.meliloti* 220-5 while *R.meliloti* 2011 can. The iron nutrition bioassays also confirmed the findings of the catechol and hydroxamate assays as it was also shown that *R.meliloti* 220-5 could not use hydroxylamine, 2,3 DHBA or anthranilate as sources of complexed iron. Table 3.9.

			Test	Solut	tion*		
		(grow	th m	easure	ed in	mm)	
A	B	С	D	E	F	G	H
~	-	260		-	350	-	-
+	1.2	250	÷	-	300	-	
÷	÷.,	260	+	-	230		-
~	-	300	-		350	110	80
÷	-	300		÷	250	150	70
	A - - - -	<u>A</u> B 	(grow <u>A B C</u> 260 250 260 300 300	Test (growth m <u>A B C D</u> - 260 - - 250 - - 260 - - 300 - - 300 -	Test Solut (growth measure <u>A B C D E</u> 260 - 250 260 - 300 - 300	Test Solution* (growth measured in A B C D E F - - 260 - 350 - - 250 - 300 - 260 - 230 - 260 - 230 - 300 - 350 - 300 - 250	Test Solution* (growth measured in mm) A B C D E F G - - 260 - 350 - - - 250 - 300 - - - 260 - 230 - - - 300 - 350 110 - - 300 - 250 150

* Hydroxylamine (A), anthranilate (B), desferal (C), 2,3 DHBA (D), CAA (E), Fe (F), concentrated supernatant (24 hours) (G), concentrated supernatant (60 hours) (H).

3.5. Isolation of rhizobactin 220-5

Rhizobactin 220-5 was isolated following the procedure for the isolation of rhizobactin from R.meliloti DM4 described by Smith and Neilands (1984). A single colony of R.meliloti 220-5 was used to inoculate 5ml TY broth. After reaching stationary phase, 1ml of this culture was used to inoculate 8 x 250ml flasks of low iron Tris medium. The flasks were shaken at 30° C, 150rpm for 48 hours. After removal of the cells by centrifugation the supernatant freeze-dried was and concentrated approximately 10-fold. Approximately 0.5g/L FeSO, .7H, 0 was added to the yellow culture supernatant. The resultant orange solution was brought to >95% saturation with ammonium sulphate (0.65 Kg/L) and the pH adjusted to 5.1 with concentrated HCl. The solution was extracted with approximately 1/5 volume of phenol:chloroform [1.0:1.5 (w/w)] and the orange organic extract was filtered through two layers of Whatman No 1 filter paper, diluted with 6 parts diethylether, and the orange colour completely back-extracted into water. The back-extract was washed with diethylether to remove traces of phenol. The crude extract containing ferrated rhizobactin was concentrated to dryness by lyophilisation and redissolved in 0.2M pyridine and adjusted to pH 6.4 with glacial acetic acid. The solution

was chromatographed on a column (18cm x lcm) containing DEAE-Sephadex-C-25 equilibrated with 0.2M pyridine-acetic acid, pH 6.4. Fractions containing siderophore were monitored by a spot assay using Hathway's reagent (2.6) (Fig. 3.6.) On DEAE-Sephadex, the solution split into two fractions; the major fraction (A) was not retarded on the column while the other fraction (B) was retained and was eluted with 0.8M pyridine pH 5.3. Both fractions were bioactive as determined by iron nutrition bioassays. Fractions A and B were freeze-dried and resuspended in 0.2M pyridine, pH 5.3. The fractions were chromatographed on a column containing Bio-Gel P2 (200-400 Mesh, Biorad laboratories) equilibrated in 0.2M pyridine pH 5.3. Fraction A gave rise to a front running minor fraction C and a major fraction D. Both fractions were bioactive (Fig. 3.7.). Fraction B ran as a compact band. Fraction B and D were deferrated with 8-hydroxyquinolone (2.6).Deferrated rhizobactin 220-5 was converted to the free acid by passage through a column of CM-sephadex C-25 equilibrated in water. The eluates were lyophilised to dryness.



Fig 3.6

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Spot assay of individual column elutions comprising fraction A. The green colour indicates the presence of siderophore.





Bioactive samples from fractions D and C.



3.5.1 IR spectrum of rhizobactin 220-5.

Fraction D, being the largest fraction obtained in the purification procedure was analysed by IR spectroscopy. An infrared spectrum is a highly characteristic property of an organic compound and can be used to establish identity of compounds and to reveal the structure of new compounds. Changes in vibrations of a molecule are caused by absorption of infrared light and a particular part of the spectrum is referred to by its frequency which is expressed in wavenumbers, cm⁻¹ or reciprocal centimetres. A particular group of atoms gives rise to characteristic absorption bands, i.e. a particular group absorbs light of certain frequencies that are much the same from compound to compound. For example, the O-H group of alcohols absorbs strongly at 3200-3600cm⁻¹; the C=0 group of ketones at 1710cm⁻¹ and the CH group at 1450 and $1375 \,\mathrm{cm}^{-1}$.

The IR spectrum was obtained on a Perkin Elmer 983G Infrared spectrophotometer using potassium bromide (KBr) disks.IR spectra for 2,3 DHBA and hydroxylamine were also obtained. Fig. 3.9. shows the IR spectrum of ferrated rhizobactin 220-5, 2,3 DHBA and hydroxylamine HC1.



Fig. 3.9

IR spectrum of ferrated rhizobactin 220-5

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Fig. 3.9 (contd.)

IR spectrum of 2,3-Dihydroxybenzoic acid

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Fig. 3.9 (contd.)

IR spectrum of Hydroxylamine

Structural information from the infrared spectra: Low frequency region $(1000-625 \text{cm}^{-1})$

Aromatic C-H bending gives strong absorptions below 900cm⁻¹. In the IR spectrum of 2,3 DHBA, an aromatic compound, the presence of four absorption bands between 900-600cm⁻¹ confirms the aromatic nature of the compound. Hydroxylamine HCl, which is not an aromatic compound lacks absorption bands in this region. The same is true of the IR spectrum of ferrated rhizobactin 220-5. The lack of absorption bands in the 1000-625cm⁻¹ region show that this compound is not aromatic, confirming the findings of the Arnow and Rioux assays for catechols.

High frequency region (4000-1350cm⁻¹)

In this region a strong broad band at ~ 3400cm^{-1} indicates O-H stretching, while a weaker, sharper band indicates N-H stretching. In the three IR spectra, there are O-H stretches at approximately 3400cm^{-1} . For hydroxylamine, the N-H stretch that should occur in this region is being masked by the strong O-H stretch of the hydroxyl group. However the strong band at 1600cm^{-1} in the hydroxylamine spectrum indicates the N-H stretching. The strong absorption band at 1679cm^{-1} in the IR spectrum of 2,3 DHBA is indicative of a C=O group attached to an aromatic group. Absorption bands of this frequency are missing in the IR spectra of hydroxylamine and rhizobactin 220-5.

The finger-print region (1350-1000cm⁻¹)

Two substances that have identical infrared spectra are, in effect, identical and must almost certainly be the same compound. One region of the IR spectrum (1350-1000cm⁻¹) is called, appropriately, the finger-print region.

The lack of bands in the aromatic region of its IR spectrum confirms that rhizobactin 220-5 is not a catechol. That it is not a hydroxamate is demonstrated by the difference in the finger-print regions of the IR spectra of hydroxylamine HCl and ferrated rhizobactin 220-5.

3.6 Thin-layer chromatography.

Deferrated rhizobactin 220-5 was analysed by thin layer chromatography. The solvent system used was methanol:water 9:1. The sample was spotted onto silica coated glass and allowed to dry before running in the solvent. Plates were viewed under UV light and sprayed with an iron reagent (0.1M FeCl in 10mM HC1). Under UV light two bands were visualised close together. When sprayed with the iron reagent these bands turned orange, the colour of ferrated rhizobactin. The presence of two bands indicated that the deferrated rhizobactin 220-5 preparation was not homogenous. An attempt was made to purify the sample by running it on a silica plate, viewing under UV light, scraping off the silica containing the major band and eluting this band in methanol. The procedure was successful, however, the yield of pure rhizobactin was very low and further analysis proved impossible. Fig. 3.10. shows the migration of the bands on the TLC plate.


Fig. 3.10

Migration of a sample of deferrated rhizobactin 220-5 on a TLC plate. The arrows indicate the sample application level, the position of the sample and the solvent front.

Discussion Three

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The development of the Chrome Azurol S universal chemical assay for the detection of siderophores by Schwyn and Neilands (1987) has proved to be of great value in the area of siderophore research, not only for the ability to detect siderophores independent of structure but also for investigation of the molecular genetics of siderophore systems. The assay is particularly useful when applied to the area of research concerned with siderophore production by *Rhizobium* species, considering the atypical nature of siderophores produced by *R.meliloti* strains.

In this study the CAS assay was used to test siderophore production by a number of strains belonging to different Rhizobium species. The results of the analysis presented in Table 3.1 indicate that siderophore production is strain specific in Rhizobium species. This was first indicated in a report by Smith and Neilands (1984) where only six of thirteen R.meliloti strains tested could utilize the siderophore, rhizobactin, produced by R.meliloti DM4 and again in a report by Guerinot et al. (1990) where only one of 20 strains of Bradyrhizobium tested produced the siderophore, citric acid. Table 3.2 presents the results of the analysis for the production of siderphore of 219 Rhizobium trifolii isolates from clover plants grown in four different alkaline soils. These results also suggest that siderophore production is strain specific in Rhizobium. A lower incidence of siderophore production in Rhizobium strains isolated from clover grown in soil 4, (Table 3.2) may be due to the high percentage total iron in this soil compared to the other three. Total iron, however, cannot be used as an indication of available Fe³⁺ but no reliable assay for the determination of Fe³⁺ from soil exists at the moment. Nevertheless, soil 4 could possess a higher percentage of bioavailable iron compared to the other three soils and this could account for the lower percentage of siderophore positive strains. Apart from demonstrating the strain specificity of siderophore production in Rhizobium species, the results of this analysis also suggest that the ability to produce a siderophore confers no obvious advantage

on *Rhizobium* strains regarding symbiosis, since all strains, both siderophore positive and negative, were isolated from effective nodules. Possession of a siderophore may benefit *Rhizobium* strains by aiding competition against other microorganisms in the soil and siderophore producing strains may dominate over non-producing strains as is seen with the pseudomonads. Detailed studies on the plasmid profiles of strains isolated from clover plants grown in various soils, in conjunction with their siderophore status, would have to be undertaken to determine whether or not this is the case.

The *Rhizobium* strain, *R.meliloti* 220-5 was shown to be positive for siderophore production using the CAS assay (Table 3.1). Because most of the work on the production, structure and molecular biology of siderophores in *Rhizobium* species has been carried out on *R.meliloti* strains, it was decided to examine the production of the siderophore from *R.meliloti* 220-5 in more detail.

That the growth of *R.meliloti* 220-5 is regulated by the concentration of available iron in the medium is demonstrated by the growth curves presented in Fig. 3.1. While there is only a slight increase in growth when the strain is grown at 40μ M iron as opposed to 1μ M iron, growth of the strain is severely retarded when the iron in the medium is bound by an iron chelator (EDDA) and is thus unavailable to the bacterium.

Using the Chrome Azurol S solution assay to test samples taken at three hourly intervals during the growth of R.meliloti 220-5 in low iron Tris medium it was observed (Fig. 3.2 and 3.3) that the production of siderophore commences 12-15 hours into the growth cycle, coinciding with the onset of exponential growth. Up to this point, growth of the strain was very gradual and presumably its iron requirement was being met by the low-affinity iron uptake system. At some point the iron concentration of the medium must become too low to be effectively sequestered by the low-affinity system and at this point the high-affinity uptake mechanism comes into operation. Log-phase growth can then commence.

As well as a qualitative check for the presence of

siderophores, the CAS assay solution can also be used quantitatively to study the dependence of siderophore secretion on nutrients, such as iron. Fig. 3.3. presents the results of the quantitative determination of siderophore production in a low iron medium. However, this assay is of greatest value when used simply to check for the presence of siderophore in culture supernatants.

The siderophore-containing supernatant of R.meliloti 220-5 was tested for the presence of catechol and hydroxamate groups using six colourimetric assays described in the literature. Results from the commonly used Arnow assay and the more recently developed catechol assay (Rioux et al. 1983) indicated that the siderophore was not a catechol. Testing the R.meliloti 220-5 supernatant for the presence of hydroxamate using three separate hydroxamate assays suggested that the siderophore does not belong to this class of siderophore either. Testing the supernatant with an assay for citric acid excluded citrate as the possible siderophore produced by R.meliloti 220-5. Results from the assays lead to the conclusion that the siderophore of R.meliloti 220-5 belongs to the recently discovered third class of siderophores, typified by rhizobactin from R.meliloti DM4. The siderophores of R.meliloti 1021, 102F28 and DM5 also belong to this class (Schwyn and Neilands, 1987). Thus, the production of atypical siderophores appears to be a general phenomenon among R.meliloti strains which possess a siderophore. So far, only the structure of rhizobactin from R.meliloti DM4 has been determined and it remains to be seen whether the siderophores from the other R.meliloti strains are structurally similar.

The siderophore from *R.meliloti* 220-5, called rhizobactin 220-5, was isolated according to the procedure used to isolate rhizobactin from *R.meliloti* DM4. When ferric sulphate was added to the yellow culture supernatant of *R.meliloti* DM4 the resultant fluid was aqua-green in colour (Smith and Neilands, 1984). However, the yellow supernatant fluid of *R.meliloti* 220-5 turned orange on addition of the iron compound. This suggests that rhizobactin 220-5 is different to rhizobactin from *R.meliloti* DM4. That this is the case was confirmed by

iron nutrition bioassays, the results of which are presented in Table 3.9. It was shown that R.meliloti DM4 could not utilize the siderophore from R.meliloti 220-5 to alleviate iron stress. An IR spectrum of ferrated rhizobactin 220-5 purified by passage through DEAE-sephadex and BioGel P2 confirmed that rhizobactin 220-5 was neither a catechol nor a hydroxamate. It however to predict the structure of was not possible rhizobactin 220-5 from its IR spectrum. Analysing a sample of deferrated rhizobactin 220-5 using thin-layer chromatography revealed the presence of two bands running close together. In the purification procedure rhizobactin 220-5 is breaking down into a number of fractions. If some of these fractions represent breakdown products of the siderophore one would imagine that they should lose some, if not all, of their bioactivity. However, bioassay results indicate that a11 fractions are bioactive. Hantke, (1990), has shown that when enterobactin breaks down in the culture medium yielding monomers and dimers of 2,3-dihydroxybenzoyl serine, these products are still capable of transporting iron. It is possible that rhizobactin 220-5 is breaking down in the culture medium into components still capable of transporting iron. This would explain fractions during the division into observed purification as well as the bioactivity seen in iron nutrition bioassays.

It is also possible that two forms of the siderophore exist, one more highly charged than the other, at the particular pH of the elution buffer used in the purification procedure. In ion exchange chromatography the more highly charged the molecule to be exchanged, the tighter it binds to the exchanger and the less readily it is displaced by other ions. In the isolation procedure presented in Fig.3.8 it would appear that fraction B is more highly charged than fraction A as it was retained on the anion exchanger and had to be eluted with a buffer of higher concentration and lower pH. That a siderophore can exist in two forms is not uncommon as has been shown for the siderophore of *Aeromonas hydrophila*. Preparations of the isolated siderophore were shown to be mixtures of two closely

related forms of the siderophore composed of 2,3-dihydroxybenzoic acid, lysine, glycine and either phenylalanine or tryptophan (Barghouti et al.1989). Thus although a partial characterization of the siderophore from R.meliloti 220-5 was possible, complete characterization regarding its structure was not, due largely to the behaviour of the crude siderophore in the purification procedure. The purification procedure needs to be reexamined and possibly modified.

Chapter Four

Identification of a gene or part thereof involved in the biosynthesis of rhizobactin 220-5.

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4.1. Isolation of Siderophore negative mutants of

R.meliloti 220-5 and R.meliloti 2011.

The RP4-specific mob-site inserted into the unique Bam Hl site of the 5,400 base-pair long transposon Tn5 was used to construct Tn5-mob, a mobile mob-site linked to an easily selectable resistance marker (Kanamycin/Neomycin) (Simon R. 1984).

Mutagenesis of *R.meliloti* 220-5 and 2011 was effected via conjugation (2.11) between *E.coli* 17-1 carrying the Tn5-mob carrier replicon pSUP 5011 and the recipient strains. Rifampicin resistant mutants of both strains had been previously isolated (2.10) and transconjugants were selected on TY agar plates containing rifampicin (100μ g/ml) and neomycin (60μ g/ml).

Colonies from selective plates were transferred onto Chrome Azurol S agar plates containing neomycin $(60\mu g/ml)$. Out of a screen of approximately 5,000 colonies for *R.meliloti* 220-5 and 2,500 colonies for *R.meliloti* 2011, four siderophore defective mutants were isolated for each strain. Absence of an orange halo surrounding these colonies, characterised the mutants as being defective in the biosynthesis of the native siderophore (Fig. 4.1.). Regulatory and transport mutants, characterised by the existence of large orange haloes in the presence of iron and large orange haloes absent in the presence of iron respectively, were not detected.



Fig 4.1

Detection of mutants defective in siderophore production using the chrome azurol S plate assay.

Mutants were further characterised as being defective in siderophore production by comparing the concentration of siderophore produced by the wild type grown in low iron Tris medium compared to that produced by the mutant, using the Chrome Azurol S solution assay (2.6).

One siderophore defective mutant of *R.meliloti* 220-5, *R.meliloti* 220-5-1, was analysed in this manner. Results showed that over a 24 hour period no siderophore was produced by the mutant while siderophore was produced by the wild type (Fig. 4.2.).

Growth of the wild type *R.meliloti* 220-5 and the mutant 220-5-1 was also compared on iron-restricted medium. The iron-chelator 2,2' Dipyridyl (20mM as an ethanol stock) was added to iron deficient Tris medium at five different concentrations. Single colonies of both wild type and mutant were streaked on the iron restricted medium and the growth scored (Table 4.1.). Table 4.1.

Concentration of	Growth of	Growth of
2,2'Dipyridyl (µM)	R.meliloti 220-5	R.meliloti 220-5-1
0	++++	++++
30	++++	++++
70	++++	++
100	** *	-
150	+	_

++++ indicates strong growth; - indicates no growth.

Results show that under iron limiting conditions, the growth of the mutant 220-5-1 is significantly retarded with respect to the wild type 220-5 indicating a defect in the production of siderophore and in iron acquisition.

Finally, the mutant 220-5-1 was compared to the wild type in the isolation procedure for rhizobactin 220-5 outlined

previously (3.5). Fig.4.3. shows the lack of orange colour, signifying the absence of the iron bound siderophore in the extract of the concentrated supernatant from 220-5-1 grown in low iron Tris medium.

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Comparison of Siderophore production during growth cycles of R.meliloti 220-5 and R.meliloti 220-51.

Fig. 4.2



Fig 4.3

A: Presence of ferrated rhizobactin isolated from the supernatant of *R.meliloti* 220-5.

B: Absence of the ferrated siderophore when the supernatant of R.meliloti 220-5-1 is carried through the isolation procedure.

4.2 Siderophore defective mutants contain one Tn5 insert. Total DNA was isolated from each of the four mutants of R.meliloti 220-5 and 2011 (2.15), restricted with EcoR1 and separated on an agarose gel. After southern blotting (2.18), the nitrocellulose filter was probed with $[\alpha - {}^{32}P]$ labelled pSUP5011 which carries Tn5-mob. Fig. 4.4. shows the result of the hybridisation. The four mutants of *R.meliloti* 220-5 each contain a single Tn5-mob insert. R.meliloti 220-5-1 and 220-5-4 contain a Tn5-mob insert on fragments of equal size while 220-5-2 and 220-5-3 also contain an insert on fragments of equal size but larger than the first two mutants. Results for R.meliloti 2011 show that mutants 2011-1, 2 and 3 contain a single Tn5-mob insert on fragments of unequal size. Mutant 2011-4 was shown to have two inserts. The two inserts could be two Tn5-mob inserts or it is also possible that one of the inserts is IS50, the 1,534 bp insertion sequence element that is a component of the transposon Tn5. This mutant was not used in any further analyses.



Fig.	4.4	1.	R.meliloti	220-5-1
		2.	R.meliloti	220-5-2
		3.	R.meliloti	220-5-3
		4.	R.meliloti	220-5-4
		5.	R.meliloti	2011-1
		6.	R.meliloti	2011-2
		7.	R.meliloti	2011-3
		8.	R.meliloti	2011-4

4.3. Cloning of the Tn5-mob containing fragment from R.meliloti 220-5-1.

Estimating from the result shown in fig.4.4, the size of the Tn5-mob containing fragment of R.meliloti 220-5-1 was known to be approximately 8-9Kb. Total DNA from this strain was restricted with EcoR1 and ligated to EcoR1 restricted pUC19 DNA in a ratio of 10:1 for insert to vector. E.coli JA221 was transformed with recombinant plasmids (2.12) and transformants were selected on LB agar containing ampicillin $(40\mu g/ml)$ to determine ligation efficiency and on LB agar containing kanamycin (50 μ g/ml) to isolate clones harbouring recombinant plasmids containing the Tn5-mob fragment. One such clone was isolated and plasmid DNA from this clone, pGR1, was prepared by the cleared lysate method (2.14) and restricted with EcoR1. The size of the Tn5-mob containing fragment was estimated as 9.2Kb (Fig.4.5.). This fragment was isolated from an agarose gel using the gene-clean procedure (2.17) labelled with $[\alpha^{-32}P]$ and used to probe total DNA digests (EcoR1) of both R.meliloti 220-5 and R.meliloti 220-5-1. Fig.4.6 shows that in R.meliloti 220-5 the mutated gene or part thereof is carried on a fragment of approximately 2Kb, while in the mutant this fragment is 9.2Kb due to the insertion of Tn5-mob.



Lane 1: pGR1 restricted with EcoR1

Fig. 4.5 Plasmid pGR1 restricted with EcoR1.



Fig. 4.6 Hybridisation of 32 P-labelled pGRl to total DNA from *R.meliloti* 220-5 and *R.meliloti* 220-5-1 restricted with *Eco*Rl.

R.meliloti 220-5
R.meliloti 220-5-1
1 kb λ ladder.

4.4 Presence of the siderophore biosynthesis gene of *R.meliloti* 220-5 in other *Rhizobium* strains.

Total DNA from nine *Rhizobium* strains was isolated, restricted with *ECoRl*, separated on an agarose gel and southern blotted. The 9.2Kb fragment from *R.meliloti* 220-5-1 was labelled and used in a hybridisation reaction to the restricted DNA from the nine *Rhizobium* strains Fig., 4.7 and 4.8.

It was evident from the autoradiographs that the gene mutated in *R.meliloti* 220-5 is present in *R.meliloti* 220-3 and *R.meliloti* 2011 but absent in the other strains tested. The two bands in the lane corresponding to *R.meliloti* 65c are due to the Tn5 transposon and IS element present in this strain. No bands were observed in the lane corresponding to *R.meliloti* 65. Results are summarized in Table 4.2.

Table 4.2.

		Siderophore	Presence of the 220-5
Rhizobium strain		production	siderophore gene.
R.meliloti	220-5	+	+
	65	1.0-01	
	65c	-	4
	220-3	+	+
	DM4	+	1.0-0
	102F34	-	-
	2011	+	+
R.legumino	sarum		
biovar viv	iae		
	J1300	+	
	3855	1.24	



LANE 1. 1 kb λ ladder

- 4. R.meliloti 220-5-1
- 5. R.meliloti 65c
- 6. R.meliloti 65
- 7. R.meliloti 220-3
- 8. R.meliloti DM4
- 9. R.meliloti 102F34
- 10. R.meliloti 2011
- 11. R.leguminosarum J1300

13. 1 kb λ ladder

Fig. 4.7 Total DNA from eight *Rhizobium* strains probed with ³²P-labelled pGR1



Lane 1: *R.meliloti* 2011 2: *R.leguminosarum* 3855 3: *R.meliloti* 220-5-1

Fig. 4.8 Total DNA from *R.meliloti* 2011, *R.meliloti* 220-5-1 and *R.leguminosarum* 3855 probed with ³²P-labelled pGR1

4.5 Complementation of the mutation in R. meliloti 220-5-1. Based on the above results, a gene bank of R.meliloti 2011 made in the cosmid vector pSUP205 kindly donated by Dr. A. Pühler, Bielefeld, was used to complement the mutation in R.meliloti 220-5-1. The method of in situ hybridisation of bacterial colonies was used (2.22). A total of 300 individual colonies of the gene bank were dotted onto nitrocelluose filters on agar plates containing tetracycline $(20\mu g/ml)$ and allowed to grow overnight at 37°C. In each case a master plate was prepared. The host carrier of the pSUP205 based gene bank is E.coli CSH56 which also harbours the mobilising plasmid RP4. In order to prepare a probe for the colony hybridisation, the mob-site in the 9.2Kb of the recombinant plasmid pGRl had to be removed to avoid hybridisation with mob sites on pSUP205 and RP4. Plasmid pGR1 was restricted with EcoR1 (to cut the fragment out of the pUC19 vector) and with BamH1 to cut out the mob-site which was originally cloned into the unique BamH1 site of the transposon In5 to construct In5-mob (Simon 1984). Thus two fragments between 3-4Kb of the original 9.2Kb fragment were isolated from a gel (2.17) (Fig 4.9), labelled and hybridised to the colony blots, Fig. 4.10. The hybridisation identified two cosmids binding to the labelled probe. The intensity of hybridisation was stronger for cosmid 3 than for cosmid 7.

Cosmids 3 and 7 along with a non-hybridising cosmid, cosmid 2, were mated via conjugation into mutant 220-5-1. Initially transconjugants were selected on TY agar containing rifampicin $(100\mu g/ml)$ and tetracyline $(20\mu g/ml)$. A single transconjugant from each mating was selected and streaked on CAS agar plates containing neomycin $(60\mu g/ml)$ and tetracycline $(20\mu g/ml)$. Results (Fig. 4.11) show that cosmid 3 complements the mutation in *R.meliloti* 220-5-1 but no completmentation was observed with cosmids 2 and 7.

Cosmids 2, 3, 7 and seven other non-hybridising cosmids were restricted with *Eco*Rl, separated on an agarose gel, southern blotted and probed with the labelled fragments described above (Fig. 4.12).



Lane 1: pGR1 restricted with BamH1 and EcoR1. Lane 2: 1 kb λ ladder.

Fig. 4.9 Fragments of pGR1 labelled for colony hybridisations.



JA221 containing pGR1

cosmid 3

Fig. 4.10 Hybridisation of ³²P-labelled pGR1 to colony blots of *E.coli* CSH56 harbouring cosmids of the *R.meliloti* 2011 gene bank.





R.meliloti 220-5-1 before complementation with cosmid 3.

R.meliloti 220-5-1 after complementation with cosmid 3.

Fig. 4.11 Complementation of the mutation in R.meliloti 220-5-1 with cosmid 3



R.meliloti 220-5-1 after complementation with cosmid 7.

Fig. 4.11 (contd.)

		. 11	×	1
	3 4 5	678	9 10	
-	-	-	*	•
:	-			-

Lane	1:	1kb λ 1	adder	7:	cos	nid	6
	2:	cosmid	1	8:	cos	id	7
	3:	cosmid	2	9:	cosn	nid	8
	4:	cosmid	3	10:	cosn	id	9
	5:	cosmid	4	11:	cos	id	10
	6:	cosmid	5	13:	1 kb	λ	ladder

Fig. 4.12 Hybridisation of 32 P-labelled pGRl to 10 cosmids (one complementing the mutation in *R.meliloti* 220-5-1) from the *R.meliloti* 2011 gene bank

The hybridisation identified a 2Kb fragment in cosmid 3 hybridising very strongly to the labelled fragments of pGR1. A 3kb band hybridising to the cloned fragmnt could possibly be explained by reiteration of the sequence on another fragment.Cosmid 7, which does not complement the mutation in R.meliloti 220-5-1, has an identical hybridisation pattern to cosmid 3 apart from the 2Kb fragment. This result along with those presented in figs. 4.6, 4.7 and 4.8 suggest that it is carries the 2Kb fragment which the genomic sequence complementing the mutation. The band appearing between 8-9Kb in all of the cosmids except cosmid 6 is due to hybridisation between the cos site of pSUP205 and that of the lambda ladder labelled with the pGR1 fragments in the random priming reaction.

Cosmid 3 was mated into the seven other mutant strains to check for complementation. Results of this complementation analysis are shown in Table 4.3.

Table 4.3.

	Сотр	lementation Si	ize of the EcoRl fragment
Mutant strain		by cosmid 3	containing Tn5-mob.
R.meliloti 220-5-1		+	9.2КЪ
	220 - 5-2	+	~10.6
	220-5-3	N.D	~10.6
	220-5-4	+	9.2
R.meliloti	2011-1	+	~8.0
	2011-2	+	~10.6
	2011-3	N.D	N.D

N.D= not determined.

The complementation data indicates that cosmid 3 carries more than one gene coding for siderophore biosynthesis.

Rhizobium meliloti 102F34 does not produce a siderophore. When cosmid 3 was mated into this strain and the resulting transconjugants streaked on CAS agar it was observed that 102F34 could now produce a siderophore, (Fig.4.13). It is not known whether cosmid 3 contains a genomic sequence coding for a signal that switches on latent siderophore genes in 102F34 or whether cosmid 3 possesses the entire complement of genes necessary for the synthesis of the *R.meliloti* 220-5 siderophore.

4.6 A possible strategy for the isolation of the gene coding for the outer membrane receptor for the iron-siderophorecomplex of R.meliloti 220-5.

In this study it was not possible to isolate transport mutants of either of the two *R.meliloti* strains by random Tn5 mutagenesis. Having observed in bioassays that *R.meliloti* 102F34 cannot utilize rhizobactin 220-5 to reverse iron starvation, it was decided to exploit this fact and the fact that 102F34 does not produce its own siderophore to attempt to isolate the gene coding for the outer membrane receptor of *R.meliloti* 220-5.

The *R.meliloti* 2011 gene bank was mated *en masse* into *R.meliloti* 102F34. Transconjugants were selected on TY agar containing rifampicin $(50\mu g/ml)$ and tetracycline $(20\mu g/ml)$, washed off into 5ml of sterile water and a 10^{-4} dilution plated onto Tris medium containing tetracycline $(20\mu g/ml)$, EDDA $(100\mu g/ml)$ and 50ml/L of the siderophore-containing culture supernatant of *R.meliloti* 220-5 which had been filtered sterilised. A 10^{-4} dilution of a culture of *R.meliloti* 102F34 plated onto the same medium was used as a control. After four days some background growth had appeared on both the control and test plates but approximately fifty large colonies had grown on the test plates.

Theoretically, because *R.meliloti* 102F34 cannot use rhizobactin 220-5, the transconjugants growing on the medium containing siderophore should harbour a cosmid carrying the gene for the outer membrane receptor enabling uptake of the iron-siderophore complex. However, in repeated bioassays it could not be demonstrated that rhizobactin 220-5 reversed iron starvation of *R.meliloti* 102F34 in iron-limiting medium. It was concluded that the gene for the outer membrane receptor was not carried

on any of the cosmids harboured by the transconjugants.

When *R.meliloti* 102F34::cos 3 was tested in bioassays to check if cosmid 3 carried the gene for the outer membrane receptor no reversal of iron starvation by rhizobactin 220-5 was observed. It remains to be determined whether the cosmids isolated as described above are identical to cosmid 3 and also whether the siderophore produced by *R.meliloti* 102F34::cos 3 and the transconjugants above is rhizobactin 220-5.



Fig. 4.13 *R.meliloti* 102F34 harbouring cosmid 3. 4.7 Plant tests.

Plant tests were carried out on both the wild type and mutants of *R.meliloti* 220-5 and 2011 to determine what effect, if any, the mutation in siderophore production had on symbiosis. Two varieties of *Medicago sativa*, du Poits and Gemini, were used. The plant test medium was prepared as described (2.3) except that for some tests iron was excluded from solution 1. Seeds were germinated at room temperature in the dark for 2-3 days, inoculated with the appropriate culture and allowed to grow for four weeks at 20°C. The results of the plant tests are presented in Table 4.4 and Fig. 4.14.and 4.15.

Table 4.4

Rhizobium	No. of	Nodule	Plant	Iron	Plant
strain	nodules	type	colour	+ -	type
R.m 2011	2	pink	green	+	Gem.
2011-1	2	pink	green	+	Gem.
2011-2	2	pink	green	+	Gem.
2011	3	pink	green	-	duP.
2011-1	2	pink	green	-	duP.
2011-2	1	pinkish	greenish	-	duP.
R.m 220-5	2	small,white	greenish	+	Gem.
220-5-1	5	white	greenish	+	Gem.
220-5-2	5	white	greenish	+	Gem.
220-5-4	4	pinkish	greenish	+	Gem.
220-5	2	small,white	chlorotic	-	Gem
220-5-1	1	pinkish	chlorotic	-	Gem
220-5-2	-	-	chlorotic	-	Gem.
220-5-4	4	white	chlorotic	-	Gem.
220-5	5	white	green	÷	duP.
220-5-1	2	pink	green	+	duP.
220-5-2	10	small,white	chlorotic	+	duP.
220-5-4	1	pink	green	+	duP
220-5	-	-	chlorotic	-	duP.
220-5-1	-	-	chlorotic	-	duP.
220-5-2	-	-	chlorotic	-	duP.
220-5-4	-	-	chlorotic	-	duP.
Uninoculated	đ				
control	-	-	green	+	Gem.
Uninoculate	đ				
control	-		chlorotic	-	Gem.





R.meliloti 2011 Gemini +Fe R.meliloti 2011 Du Puits - Fe

Fig. 4.14



R.meliloti 2011-2 Gemini +Fe

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R.meliloti 2011-2 Du Puits -Fe

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Fig. 4.14 (contd.)


R.meliloti 220-5 R.meliloti 220-5-2 R.meliloti 220-5 R.meliloti 220-5-2 Gemini +Fe Gemini +Fe Gemini -Fe Gemini -Fe

Fig. 4.15



R.meliloti 220-5-4 R.meliloti 220-5-1 Gemini -Fe Gemini -Fe

Fig. 4.15 (contd.)



R.meliloti 220-5 R.meliloti 220-5-2 R.meliloti 220-5 R.meliloti 220-5-2 Du Puits +Fe Du Puits +Fe Du Puits -Fe. Du Puits -Fe Regarding *R.meliloti* 2011, results of the plant tests indicate that there is no appreciable difference between the wild type strain and the mutants concerning the formation of nodules. Both wild type and mutants, used to inoculate plants grown on high and low iron medium, elicit nodule formation. Plants inoculated with *R.meliloti* 2011-1 and 2011-2 do exhibit some chlorosis, however, compared to the wild type, *R.meliloti* 2011, and nodules are slightly smaller and less pink.

Lack of iron in the plant medium results in chlorotic plants for both the wild type and mutant strains of R.meliloti 220-5 as well as the development of small white nodules. When iron is added to the plant medium the plants are healthy and nodules formed improve somewhat with regard to pink colouration and therefore leghaemoglobin content. However the nodules formed by R.meliloti 220-5 are smaller and much less pink compared to those formed by R.meliloti 2011 and some incompatibility with the host plant may be the reason. It was also observed that R.meliloti 220-5 wild type and mutant strains failed to nodulate the Du Puits variety of Medicago sativa in the absence of iron. For the Gemini variety this failure to nodulate in the absence of iron this mutant elicited the formation of a large number (10) of small, white nodules on the Du Puits plants. Discussion 4

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The CAS assay was used as a primary screen to facilate isolation of mutants of R.meliloti 220-5 and 2011 defective in some aspect of their iron assimilation systems. It proved defective relatively easy to isolate mutants in the biosynthesis of the native siderophores but it was not possible to isolate either regulatory or transport mutants despite screening over 5,000 Tn5-containing strains for R.meliloti 220-5 and approximately 2,500 for R.meliloti 2011. This difficulty in isolating transport mutants was also observed by Gill and Neilands, (1989). Having screened a large number (16,000) of Tn5-containing strains to increase the probability of obtaining a complete collection of iron assimilation mutants they identified a class of mutants having a phenotype typical of transport mutants. When this class of mutants was further analysed it was found that the mutants lacked none of the low-iron-induced outer membrane proteins produced by the wild type strain and were not analysed further.

Apart from their phenotype on CAS blue agar plates, mutants in this study were characterised as being defective in siderophore production using the CAS solution assay. One mutant analysed, R.meliloti 220-5-1, showed no production of its siderophore over a 24 hour period growing in iron-limited medium. This mutant also showed significantly restricted growth on iron chelator medium but growth was not totally restricted. This could be a consequence of a low affinity iron-uptake system operating in R.meliloti 220-5. Such a low-affinity system must exist in R.meliloti 102F34 since it was observed that, despite not producing its own siderophore, this strain grew almost as well as R.meliloti 220-5 in low iron Tris medium (data not given). Thus it can be envisaged that the low-affinity pathway of iron uptake functions until it becomes impossible to sequester additional iron, at which point the high affinity mechanism is switched on. In R.meliloti 220-5-1 this high affinity system is mutated and therefore growth of the bacterium is retarded under conditions of iron stress. Another explanation could be that R.meliloti 220-5-1 is producing a functional amount of siderophore, but in amounts too low to be

detected in the CAS solution assay or to produce haloes on CAS blue agar plates.

fragment containing the Tn5-mob The EcoRl mutation in R.meliloti 220-5-1 was subcloned into pUC19 and purified after digestion using the gene clean procedure. This fragment was then used as a probe to detect the presence of the mutated siderophore biosynthesis gene in other Rhizobium strains. The presence of the gene in R.meliloti 2011 and its absence in R.meliloti DM4 is not unexpected since it was observed in bioassays that R.meliloti DM4 could not utilize rhizobactin 220-5 under conditions of iron stress and must therefore possess a different siderophore to R.meliloti 220-5. R.meliloti 2011 was found to be capable of utilizing the siderophore of R.meliloti 220-5 suggesting that these strains possess very similar, if not identical, siderophores. The absence of the gene in R.meliloti 65 and 102F34 is not surprising considering that neither of these strains appear to produce a siderophore. That some R.meliloti strains carry the gene while others do not presents further evidence for the strain specificity of siderophore production in Rhizobium.

The presence of the R.meliloti 220-5 siderophore biosynthesis gene in R.meliloti 2011 permitted the use of a gene bank of R.meliloti 2011 to complement the mutation in R.meliloti 220-5-1. A single cosmid complementing the mutation was isolated from the gene bank and when analysed for its ability to complement the other R.meliloti 220-5 and 2011 mutant strains, complementation data indicated that cosmid 3 carried more than one siderophore biosynthesis gene. It is likely that the genes required for siderophore biosynthesis are clustered in the genome of *R.meliloti* 220-5 as has been observed for the enteric bacteria, pseudomonads and more relevantly for R.meliloti 1021 (Gill and Neiland, 1989). That cosmid 3 confers on R.meliloti 102F34 the ability to produce a siderophore may be an indication that cosmid 3 carries all the genes necessary to synthesize rhizobactin 220-5. To check whether or not this is the case it will be necessary to isolate the siderophore produced by R.meliloti 102F34::cos 3 in order to determine if it is rhizobactin 220-5. Site-directed mutagenesis of cosmid 3, to be undertaken in the future, will indicate the exact number of genes carried on this cosmid.

The strategy outlined for cloning the outer membrane protein involved in uptake of the siderophore-iron complex was inspired by a similar strategy described by Magazin et al., 1986. It cannot be explained why cosmids of the R.meliloti 2011 gene bank, harboured by R.meliloti 102F34 transconjugants growing on rhizobactin 220-5 appear not to carry the gene for the outer membrane receptor. It is interesting to note however that these cosmids carry genes for siderophore biosynthesis since all transconjugants tested on CAS plates were positive for siderophore production. Considering the selective pressure placed on transconjugants to isolate cosmids carrying the outer membrane receptor gene, this result is extremely puzzling. Were it not for the results of the bioassay, it would be tempting to suggest that the gene for the outer membrane receptor is clustered with the genes for siderophore biosynthesis. It will be necessary to carry out further analysis of these cosmids.

Nodulation tests were performed on the siderophore mutants of *R.meliloti* 220-5 and 2011 to determine a possible role for the iron-assimilation system in symbiosis. Results from the plant tests indicate that for *R.meliloti* 2011 the ability to produce a siderophore confers no obvious advantage with regard to symbiosis because apart from the slight chlorosis exhibited by plants inoculated with wild type and mutants on low iron medium, mutants still elicited formation of nodules that appeared normal and healthy.

At low iron levels, nodules formed by *R.meliloti* 220-5 strains on *Medicago sativa* plants of the Gemini variety were smaller and less coloured than those formed by *R.meliloti* 2011 strains but the effect was observed with wild type and mutants alike indicating perhaps some incompatability with the host plant.

It was observed that *R.meliloti* 220-5 strains, mutant and wild type, failed to nodulate *Medicago sativa* plants of the Du Puits variety in the absence of iron. O'Hara, *et al.* (1988), found that iron-deficiency specifically limits nodule development in peanut inoculated with Bradyrhizobium species. However, nodule initiation was not affected as similar numbers of nodule initials formed in the roots of control and iron sprayed plants. That iron deficiency can totally inhibit nodule formation is not uncommon as it has been observed that total nodulation failure is oftenrecorded on certain peanut cultivars growing in the iron deficient calcareous soils of Thailand. The report by O'Hara et al. also highlighted the fact that some cultivars can be more susceptible to iron deficiency than others. Since any strain nodulating a plant becomes subject to the iron status of that plant, it can be expected that nodule initiation and/or development will be inhibited to a greater extent in plants with a greater susceptibility to iron deficiency. Regarding the observation made in this study, it could be argued that the Du Puits variety of Medicago sativa is displaying a greater susceptibility to iron deficiency than the Gemini variety thus inhibiting nodule formation by depriving the nodulating strain of sufficient iron. However, results from plant tests with R.meliloti 2011 would appear to discount this argument since both wild type and mutant strains form normal nodules on Du Puits plants grown on low iron medium. Nevertheless, R.meliloti 220-5 is displaying some incompatibility with its host plants and this, along with the low iron stress imposed on the plant could lead to the inhibition of nodule formation for this strain.

The behaviour of the mutant *R.meliloti* 220-5-2 in the plant tests is interesting to note since this mutant carries the Tn5-mob insert on a larger fragment, (~10.6 Kb), than either mutants 220-5-1 or 220-5-4, (9.2 Kb). It appears that the mutation in *R.meliloti* 220-5-2 is having a more marked effect on nodulation than is observed for the other mutants. Further analysis of this mutant needs to be undertaken.

It can be concluded from the plant tests with *R.meliloti* 2011 that lack of a siderophore does not inhibit nodule formation. The results from plant tests with *R.meliloti* 220-5 strains are harder to interpret but appear to indicate that the level of iron available to the plant is crucial in determining

nodulation efficiency.

The iron-transport system studied is functional in the free-living bacterium but it is not known whether it would be functional in bacteroids. It is interesting to note that to date, plant tests carried out to determine a possible role for the iron assimilation system of R.meliloti in symbiosis have looked at mutants deficient in siderophore production only. In order to discount a role for iron assimilation in symbiosis, plant tests must be carried out on mutants defective in transport of the iron-siderophore complex. Recently, Nadler et al. (1990), described a Rhizobium leguminosarum mutant defective in symbiotic iron acquisition that was apparently mutated in a gene encoding or regulating formation of a cell-bound element of a specific iron acquisition system. The authors suspect that the strain is defective in iron anthranilate metabolism, anthranilate being the native siderophore of the R.leguminosarum strain.

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Chapter Five

Outer membrane proteins induced by low iron in *R.meliloti* 220-5

5.1. Introduction

In all siderophore mediated high-affinity iron uptake systems studied in bacteria it has been demonstrated that under conditions of iron starvation, outer membrane proteins are induced in order to facilitate uptake of the iron-siderophore complex. In this chapter it is demonstrated that iron-induced outer membrane proteins are present in *R.meliloti* 220-5 under such conditions.

5.2. Low-iron-induced outer membrane proteins of R.meliloti 220-5.

Outer membrane proteins of R.meliloti 220-5 were prepared according to the method already described (2.23). In the initial experiment outer membranes were prepared from cultures of R.meliloti 220-5 that had been allowed to grow for approximately 55 hours. Fig. 5.1. shows the outer membrane protein profile of R.meliloti 220-5 grown in different iron concentrations. Under iron-limiting conditions there is induction of two outer membrane proteins, 78,000 da. and 72,000 daltons in size. The size of the proteins was calculated by reading the Rf values (calculated as distance travelled/ length of the gel) of the induced proteins from a graph of the Rf values of the marker proteins (Fig.5.2). Outer membranes were also prepared from cultures of R.meliloti 220-5 that had been growing for 24 and 39 hours (Fig. 5.3). Again, the appearance of the low-iron-induced outer membrane proteins is seen.



Fe 40 μM
 Fe <1 μM
 Fe <<1 μM
 30μg/m1 EDDA
 30μg/m1 EDDA

Fig. 5.1 Outer membrane profile of *R.meliloti* 220-5 grown in different iron concentrations.



Calibration curve

Relative Mobility (Rf)

Fig. 5.2 Graph of Rf values of standard molecular weight markers



Fig. 5.3 Outer membrane protein profiles of *R.meliloti* 220-5 prepared at 24 and 39 hours into the growth cycle.

Fe 1μM (24 hours)
 Fe 40μM (24 hours)
 Fe <1μM (24 hours)
 Fe 40μM (39 hours)
 Fe <1μM (39 hours)
 EDDA 30μg/m1 (55 hours)

5.3. Low-iron-induced outer membrane proteins are present in the mutant *R.meliloti* 220-5-1.

The phenotype of *R.meliloti* 220-5-1 on CAS agar plates indicates that it is only defective in the biosynthesis of rhizobactin 220-5. As such it is expected that *R.meliloti* 220-5-1 should still induce the production of the low-iron-induced outer membrane proteins under iron restricted conditions. Outer membrane proteins were prepared from 24 hour cultures of *R.meliloti* 220-5-1 grown in the presence and absence of iron. In Fig. 5.4. the outer membrane proteins induced when iron is limiting are shown to be present in the mutant.



Fig. 5.4 Outer membrane protein of *R.meliloti* 220-5-1 grown under iron replete and deficient conditions.

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Fe < 1μM (24 hours)
 Fe 1 μM (24 hours)
 Fe 1 μM (24 hours)

5.4. Polyclonal antibody to the low-iron-induced outermembrane proteins from *R.meliloti* 220-5.

In this study, Tn5-mob mutagenesis of *R.meliloti* 220-5 failed to identify a mutant lacking any of the low-iron-induced outer membrane proteins. The strategy outlined in Chapter Four (Section 4.) also seems to have failed in identifying the gene coding for the outer membrane receptor. Therefore, it was decided to attempt to raise an antibody to the induced proteins with a view to using it in the future to screen a gene expression library of *R.meliloti* 220-5 in order to identify genes coding for these proteins.

The two low-iron-induced outer membrane proteins were cut from an SDS-polyacrylamide gel and prepared as described (2.25). Proteins from approximately ten lanes were used for each injection. On day one, the gel and adjuvant emulsion was injected subcutaneously into a rat. This was followed by two subsequent injections on day fifty and sixty four. Seven days after the final injection a small aliquot $(500\mu l)$ of blood was extracted from the tail of the rat, allowed to clot, and the serum tested for the presence of the desired antibody. On day seventy two the rat was sacrificed and approximately 5ml of blood collected. After standing at 4°C overnight to allow clotting, the blood was centrifuged at 5,000 rpm for 10 minutes and the serum collected. The serum containing the antibody (1:33 dilution) was incubated with a Western blot of the gel shown in Fig. 5.5. according to the procedure outlined (2.27). To test for non-specific binding, a duplicate Western blot was incubated with serum from a rat immunized with a different antigen to the one used in this experiment. Fig. 5.6. shows the reaction of the control serum and the test serum with the western blots. While there is some general non-specific binding when the control serum is incubated with the western blot, it is evident that the test serum contains an antibody reacting against the two low-iron-induced outer membrane proteins used to immunize the animal. There is some cross-reactivity with a non low-iron-induced protein that occurs just above the is most probably due twoinduced proteins and this to

contamination of the antigen proteins with this protein when they were being cut out of the SDS-polyacrylamide gel. The titre of the antibody is low and this is because time constraints permitted only three immunizations to be carried out whereas four or five would have resulted in a much higher titre.

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Fig. 5.5 Outer membrane protein profile of *R.meliloti* 220-5 and 102F34 used in Western blot analysis



Lane 1: R.meliloti 220-5 + Fe 2: R.meliloti 220-5 - Fe 3: R.meliloti 102F34 + Fe 4: R.meliloti 102F34 - Fe

Fig. 5.6 Western blot detection of proteins using a polyclonal antibody to low-iron-induced proteins



Lane 1: R.meliloti 220-5 + Fe 2: R.meliloti 220-5 - Fe 3: R.meliloti 102F34 + Fe 4: R.meliloti 102F34 - Fe

Fig. 5.6 (contd.) Non-specific binding of control serum to Western Blot.

Discussion Five

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Isolating the outer membrane proteins of R.meliloti 220-5 grown under iron replete and iron deficient conditions has shown the induction of two outer membrane proteins produced when iron in the medium is limiting. The molecular weights of these proteins, 78,000 and 72,000 daltons respectively, fall within the range of the molecular weights observed for low-iron-induced proteins in other species, i.e. 81,000 da. for the outer membrane receptor for ferric enterobactin and 74,000 da. for the receptor for ferric aerobactin, both induced in E.coli species; 86,000 da. for the OM2 receptor induced in Vibrio anguillarum strains to transport ferric anguibactin and 85,000 da. for the outer membrane receptor for ferric pseudobactin induced in Pseudomonas fluorescens. The demonstration that iron-related outer membrane proteins exist in R.meliloti 220-5 makes it difficult to understand why it was not possible, in a screen of over 5,000 Tn5-mob containing strains, to isolate a transport mutant. Because efforts to isolate a mutant lacking the outer membrane receptor for the iron-siderophore complex, or to isolate the gene itself, failed, it is not possible to identify which of the induced proteins is the receptor.

When the outer membrane proteins from *R.meliloti* 220-5-1, a siderophore deficient mutant of *R.meliloti* 220-5, were isolated, it was shown (Fig.5.4) that induction of the iron-related outer membrane proteins was unaffected. This was as expected since the mutant did not show a phenotype typical of a transport mutant, a large orange halo absent in the presence of iron, on CAS plates.

A polyclonal antibody was raised to the two iron-related proteins by injecting a rat, at intervals, with an emulsion of Freund's adjuvant and the gel fragments containing the induced proteins. The antibody was raised primarily with a view to using it to screen a gene expression library of *R.meliloti* 220-5 in order to identify the gene coding for the outer membrane receptor since other attempts to identify this gene were unsuccessful. It is also hoped to use the antibody to test *R.meliloti* 220-5 bacteroid

membranes for the presence of the low-iron-induced proteins observed in the outer membranes of the free-living form. It is not known at this time whether the siderophore system of iron uptake is functional in bacteroids and demonstrating absence of the R.meliloti 220-5 the presence or iron-related proteins in the bacteroid membrane would contribute significantly to answering this question. The antibody can also be used to check the outer membrane profiles of other Rhizobium strains for iron-related proteins cross-reacting with the R.meliloti 220-5 proteins. One aim was to use the antibody to probe the outer membrane proteins of the R.meliloti 102F34 strain harbouring cosmid 3 to check for the presence of the R.meliloti 220-5 iron-related outer membrane proteins which should be present if cosmid 3 carries the genes for the receptor. However, this strain grew very poorly in low iron Tris medium and sufficient quantities could not be grown to isolate the outer membranes. The antibody was used however, to probe a western blot of the outer membrane proteins of R.meliloti 102F34 grown under iron-limiting conditions (Fig.5.5). While R.meliloti 102F34 does appear to induce the production of at least one protein (Fig.5.5, lane 5) under iron stress, this protein does not react with the antibody. The production of an iron-related protein by R.meliloti 102F34 is interesting, considering that this strain does not produce a siderophore.

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