The moving finger writes and having writ, moves on.  
Nor all your pity nor wit shall bare it back to cancel half a line,  
Nor all your tears wash out a word of it.  

.........................................E Fitzgerald


- **Geetha R, Falguni Singh, Anjana J Desai, G. Archana.** “Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains along with *Rhizobium spp*” Communicated in *Bioresource Technology*. 
List of posters presented

- 44th Annual Conference of Association of Microbiologist of India (Poster Presentation), 12-14 Nov 2003 at Karnataka. Entitled: “Siderophore production and cross-utilization studies of rhizobial and non-rhizobial isolates from rhizospheric soil of Cajanus cajan”.

- 46th Annual Conference of Association of Microbiologist of India (Poster Presentation), 08-10 Dec 2005 at Osmania University, Hyderabad. Entitled: “Ferrichrome utilization by transgenic rhizobia expressing E. coli fhuA gene”.

- 3rd BRSI International Conference (Poster Presentation), 02-04 Nov 2006 at Sardar Patel University, Vallabhbh Vidya Nagar, Anand, Gujarat. Entitled: “Isolation of Nodule associated microorganisms from root nodules of Cajanus cajan and their possible role in plant growth promotion”.

- 75th Society of Biological Chemist (Poster accepted), 08-11 Dec 2006 at Jawaharlal Nehru University, Delhi. Entitled: “Rhizospheric performance of transgenic rhizobia expressing E. coli fhuA gene encoding Fe³⁺: ferrichrome receptor”.

List of award

- Awarded, first prize in poster titled “Ferrichrome utilization by transgenic rhizobia expressing E. coli fhuA gene”, in 46th Annual Conference of Association of Microbiologist of India.
Differential cross-utilization of heterologous siderophores by nodule bacteria of *Cajanus cajan* and its possible role in growth under iron-limited conditions

Arif Khan ¹, R. Geetha ¹, Aparna Akolkar, Ami Pandya, G. Archana, Anjana J. Desai *

Department of Microbiology and Biotechnology Centre, Maharaja Sayajirao University of Baroda, Vadodara 390002, Gujarat, India

Received 6 June 2005; received in revised form 22 November 2005; accepted 20 December 2005

Abstract

Siderophores are ferric specific ligands that are involved in receptor specific iron transport into bacteria. Partially purified siderophores from 37 different rhizospheric bacterial isolates, and 17 *Cajanus cajan* root nodule isolates were tested for their utilization by the 17 nodule isolates as test organisms. Significant variation in the siderophore cross-utilization pattern was observed. Catecholate siderophores were produced predominantly by nodule isolates whereas rhizospheric isolates produced catecholates, as well as hydroxamates. The quantity of hydroxamate produced was higher than that of catecholates. Considerable variation was observed with respect to siderophore cross-utilization amongst nodule isolates obtained from a particular location. Isolates collected from different nodules of same plant also showed considerable variation with respect to siderophore cross-utilization. Isolates, which showed, higher siderophores cross-utilizing ability, when tested for homologous (from other nodule bacteria) siderophores also gave good cross-utilization of heterologous (from non-nodule rhizospheric bacteria) siderophores. Isolate GHU(iii), the highest cross-utilizer of homologous and heterologous siderophores showed a significant increase in growth in the presence of exogenously added siderophores. Similar results were observed during the co-inoculation studies with rhizospheric isolate under iron-limited condition. Both isolates GAU(11) and AP(64), low cross-utilizers of siderophores, failed to show growth increase in response to co-inoculation and addition of siderophores. The iron regulated outer membrane protein (IROMP) profiles of strains showing maximum or least cross-utilization did not differ. The significance of these findings in context to survivability under natural conditions and the possible reasons accounting for high siderophore cross-utilizing status of isolate GHU(iii) is discussed.

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Keywords: Root nodule bacteria; Siderophore cross-utilization; *C. cajan*

1. Introduction

Iron is the fourth most abundant element found in the Earth’s crust and is present largely in the Fe³⁺ state
ability to produce siderophores has been demonstrated to confer a selective growth advantage to the producer organism (De Bellis and ErcoianTi, 2001). It has also been speculated that besides capturing iron quotas necessary for growth, siderophores are also a type of iron scavengers because they can bind iron from weaker ferric–siderophore complexes from other species and thus are mediators of competitive interaction among organisms. The ligand exchange mechanism in case of siderophore of *Aeromonas hydrophila* is shown to provide the bacterium with the ability to acquire iron from exogenous siderophores due to its higher affinity for iron (Stintzi et al., 2000).

Microorganisms which themselves do not synthesize a particular type of siderophore may be able to take up its iron-bound complex. The ability to cross-utilize heterologous siderophores may be accounted for, by the presence of multiple type of siderophore receptors (Cornelis and Matthijis, 2002) or use of a low specificity system that recognizes more than one type of siderophore (Crowley et al., 1991). In natural environments such as the rhizosphere, the capacity to utilize heterologous siderophores produced by other members of the rhizosphere microflora is a positive fitness factor (Raaijmakers et al., 1995; Loper and Henkels, 1999). Heterologous siderophores or its producer organism may bring about a variety of responses in other target bacterial species that are present within the same niche. Growth of some species may be inhibited (Shah et al., 1992) and this has been attributed to be one of the mechanisms by which biocontrol agents act in inhibiting the growth of pathogens in the rhizosphere (Schroeder and Baker, 1982). In certain cases, addition of heterologous siderophores results in a growth stimulatory effect (Guan et al., 2000). Presence of exogenous heterologous siderophores can induce the expression of IROMPs in target bacterial species (Crosa, 1997) and may also positively stimulate the target organism to synthesize its native siderophore (Guan et al., 2001).

Root nodule bacteria, collectively called the rhizobia, form a nitrogen-fixing symbiotic relation with their leguminous plant hosts and have a high demand for iron (Guerinot, 1991). Different rhizobia produce different types of siderophores, e.g. *Rhizobium leguminosarum* bv. *viciae*, the symbiont of peas, lentils, vetches and some beans, makes vicibactin, a cyclic trihydroxamate siderophore (Dilworth et al., 1998); *Rhizobium melliloti* makes rhizobactin which is also a hydroxamate type of siderophore (Persmark et al., 1993); catecholate siderophores are produced by rhizobia of chick pea (Roy et al., 1994) and cowpea (Modi et al., 1985; Jadhav and Desai, 1992); and citrate is the siderophore secreted by *Bradyrhizobium japonicum* (Guerinot et al., 1990). Rhizobia are also found free living in soil and siderophore production is likely to provide them with an advantage in meeting with an adequate supply of iron in the competitive soil environment. Certain rhizobia have also been shown to utilize iron bound to heterologous siderophores. *R. meliloti* DM4 can utilize the hydroxamate type siderophores, ferrichrome (made by numerous fungi) and ferrioxamine B (made by actinomycetes) in addition to its own siderophore rhizobactin (Smith and Neilands, 1984). *B. japonicum* is able to utilize Fe\(^{3+}\) bound to the two fungal siderophores rhodotorulate and ferrichrome (Plessner et al., 1993). Recently it has been shown that ability to utilize ferrichrome is important for nodule development by *B. japonicum* (Benson et al., 2005).

The present work deals with the siderophore cross-utilization by root nodule bacteria of *Cajanus cajan* (pigeon pea) belonging to the cowpea miscellany group of rhizobia. Our objective was to determine the ability of root nodule bacteria to cross-utilize siderophores produced by other root nodule bacteria of the same plant species as well as by non-nodule, rhizospheric bacteria from the roots of the same plant and another plant, i.e. *Arachis hypogea* (groundnut) in order to understand the interactions that take place amongst them in the rhizosphere.

2. Materials and methods

2.1. Bacterial strains

Bacteria isolated from the root nodules and rhizospheric soil of *C. cajan* plants obtained from the local farms were maintained on Ashby's Mannitol (AM) agar containing congo red (Jadhav and Desai, 1992). Rhizospheric bacteria of *C. cajan* and *A. hypogea* were maintained on modified AM agar in which mannitol was replaced with glucose.

The letters GAU/AP/GHU/GH used in nomenclature of strains used denote the different fields, the number or alphabet in parenthesis following that represents different plants and the last digit following that denotes isolate number.

2.2. Siderophore detection and quantification

Chrome Azurol-S (CAS) plates were used to detect siderophore production (Schwyn and Neilands, 1987). For the quantification of the siderophore produced by isolates, cultures were grown in deferrated AM broth or modified AM both for 48 h and centrifuged at 7500 × *g*
for 20 min. The culture supernatant was used to detect the type of siderophore and its quantity. Catecholate type siderophores were estimated by Arnow’s method (Arnow, 1937; Payne, 1994) using 2,3-dihydroxybenzoic acid (2,3-DHBA) as a standard. Hydroxamate type siderophores were estimated by Gibson–Magrath method using hydroxylamine hydrochloride as a standard (Gibson and Magrath, 1969; Payne, 1994).

2.3. Siderophore extraction

Siderophore extraction was done by acidifying the culture supernatant to pH 2.0 and then extracting it three times, with equal volumes of ethyl acetate. The ethyl acetate layers were pooled together and evaporated to dryness in a rotary vacuum evaporator at 79 °C (Jadhav and Desai, 1992). Extracted siderophores were then re-dissolved in normal saline and stored in vials at -20 °C.

2.4. Siderophore cross-feeding assay

AM agar plates containing minimum inhibitory EDTA concentrations were surface spread with the test organism. Wells of 8 mm diameter were bored in plates and supernatant containing 15 μg/ml siderophores of nodule isolates or rhizospheric isolates were added to the wells. FeSO₄ (100 μg/ml) and saline (pH 8.0) were added as positive and negative controls, respectively. Desferrioxamine B (Desferal, Novartis) (100 μg/ml) and ferric citrate (100 μg/ml) were added as standard hydroxamate and citrate type siderophores. Plates were incubated at 30 ± 2 °C and growth around wells (zone of growth exhibition) was monitored up to 48 h. We have utilized the term homologous siderophores for those originating from nodule bacteria regardless of whether it was the same strain or not and used the term heterologous siderophores for those obtained from the culture filtrates of rhizospheric bacteria assuming the latter to be different species and the former to be same species.

2.5. Effect of externally supplied siderophores on the growth of the organisms

Siderophores of selected root nodule isolates and rhizospheric isolates were extracted, partially purified and added (at 15 μg/ml) to the deferrated as well as the iron-supplemented (100 μM FeCl₃) AM broth. The test cultures were inoculated and absorbance at 600 nm was monitored at regular intervals during incubation at 30 ± 2 °C. Deferrated and Fe-supplemented AM broth without external siderophores were also inoculated and used as controls for comparing.

2.6. Co inoculation studies

Freshly grown cultures of GHU(iii), AP(6)4 and 88 were inoculated (10² cfu/ml) in deferrated AM broth in individual flasks. GHU(iii) and AP(6)4, both were also co-inoculated (10² cfu/ml each) with strain 88 in separate flasks of each pair. Flasks were incubated on a reciprocatory shaker at 200 rpm at 30 ± 2 °C for 36 h. Samples were drawn from each flask of every 4 h and their suitable dilutions plated on AM agar plates which were incubated at 30 ± 2 °C. The numbers of colonies observed were enumerated. In case of co-inoculated samples, root nodule bacteria and strain 88 were counted separately based on their colony morphology.

3. Results

3.1. Characterization of siderophores produced by nodule and rhizospheric isolates

Forty-eight C. cajan nodule isolates and 93 C. cajan and A. hypogea rhizospheric isolates showed siderophore production. Based on morphology, biochemical tests and quantity of siderophores produced 17 nodule isolates from plants grown on different habitats were selected for further studies. Similarly, based on unique colony characteristics as well as quantity and type of siderophores produced, 37 isolates from both the rhizospheric samples were selected for further studies. The siderophores produced by these isolates were characterized. As per the results depicted in Fig. 1, one
Table 1
Siderophore (homologous and heterologous) cross-utilization by root nodule bacteria

<table>
<thead>
<tr>
<th>Root nodule bacteria</th>
<th>Ferricitrate utilization</th>
<th>Desferrioxamine B utilization</th>
<th>Total no. of homologous siderophores cross-utilized out of 17 tested</th>
<th>Distribution of homologous siderophores utilized</th>
<th>Total no. of heterologous siderophores cross-utilized out of 37 tested</th>
<th>Distribution of heterologous siderophores</th>
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<td>Catecholate</td>
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<td>GAU(2)</td>
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<td>GAU(3)</td>
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<td>APC(1)</td>
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<td>APC(4)</td>
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<td>APC(5)</td>
<td>+</td>
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<tr>
<td>APC(6)</td>
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<td>GHU(1)</td>
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<td>GHU(2)</td>
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<td>GHU(6)</td>
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<td>05 02 24</td>
<td>07 06</td>
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<td>12</td>
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<tr>
<td>GHU(7)</td>
<td>+</td>
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<td>09 02 13</td>
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<td>07</td>
</tr>
</tbody>
</table>
3.3. Effect of exogenous siderophore supplementation and co-inoculation studies

Effect of exogenous siderophore addition on the growth of the most and least siderophore cross-utilizing C. cajan nodule isolates under iron-starved and iron-supplemented condition was studied. Data in Fig. 2 shows effect of exogenous addition of homologous siderophores from GHU(iii), GAU(1)1, GAU(3)1, AP(2)4, AP(5)4 on growth of GHU(iii) which is a better cross-utilizer and AP(6)4 which is a least cross-utilizer. Similarly the effect of exogenous addition of heterologous siderophores on growth of GHU(iii) and AP(6)4 which are proficient and deficient, respectively, on their utilization is shown in Fig. 3. Substantial increase in the growth of strain GHU(iii) in presence of externally supplied mixture of partially purified siderophores was obtained, in both the cases as compared to the least cross-utilizing isolates GAU(1)1 and AP(6)4. Exogenous addition of the same preparation of siderophores under iron-supplemented conditions failed to show any increase in growth of the isolates (data not shown).

The above results were further substantiated by the co-inoculation studies, where the growth of the most and the least siderophore cross-utilizing strain GHU(iii) and AP(6)4, respectively, were separately studied in presence of a rhizospheric strain 88 which predominantly

3.2. Siderophore cross-utilization by nodule isolates

Cross-utilizing ability of C. cajan nodule isolates was checked against homologous and heterologous siderophores. For performing the siderophore cross-utilization tests, an inhibitory EDTA concentration was incorporated in the plates which varied for different isolates and ranged from 5.5 to 8.0 mM. Nodule isolates showed considerable variation with respect to utilization of siderophores (Table 1). Isolate GHU(iii) showed the greatest cross-utilization ability with not only siderophores obtained from nodule isolates (homologous siderophores) but also those obtained from rhizospheric isolates (heterologous siderophores) (Table 1). Isolates GAU(1)1 and AP(6)4, were the poorest utilizers of homologous and heterologous siderophores. GHU(iii) utilized 40 siderophores whereas AP(6)4 could utilize only 13 and GAU(1)1 11 out of 54 tested.

The cross-utilization studies of C. cajan nodule isolates showed that 41% of the siderophores tested were being cross-utilized and amongst those 48% were homologous while 37% were heterologous siderophores. From amongst these homologous and heterologous siderophores being cross-utilized, 48% belonged to catecholate type, 19% were hydroxamate types and 34% were grouped under both categories. The results showed that C. cajan nodule isolates showed preference for catecholate over hydroxamate type siderophores. The nodule isolates showed 43% utilization of A. hypogea rhizospheric siderophores and 33% utilization of C. cajan rhizospheric siderophores showing preference for A. hypogea rhizospheric siderophores.
produced hydroxamate type siderophore. The strain GHU(iii) can utilize siderophores produced by strain 88 whereas strain AP(6)4 fails to do so. GHU(iii) showed growth stimulation in presence of 88, as compared to AP(6)4 (Fig. 4).

Since GHU(iii) showed better ability to cross-utilize homologous and heterologous siderophores, it was of our interest to compare the outer membrane protein profiles of GHU(iii) and AP(6)4 grown under iron-limited and iron-supplemented conditions. No difference in the outer membrane protein profile of the two isolates was observed and both the strains showed the induction of a protein of approximately 80 kDa mass under iron-limited condition (data not shown).

4. Discussion

The interactions between different bacterial species mediated through siderophores are of significance in the survival and functioning of species in natural environments and a thorough understanding of the basis of these interactions is important particularly in predicting the fate of bacteria such as rhizobia that are often amended to agricultural soils. Production of C. cajan was seen to increase when the alluvial soil was amended with rhizobium inoculum (Saxena et al., 1976). While siderophores are considered as an important component of bacterial machinery for iron sufficiency, in some cases, rhizobial mutants defective in siderophore synthesis, fix N₂ normally in association with their plant hosts (Reigh and O'Connell, 1993). Siderophore production by root nodule bacteria is likely to be more important for survival and growth in the competitive soil environment, which is usually deficient in soluble iron. This paper addresses the ability of native root nodule bacteria to utilize heterologous siderophores produced by other nodule as well as rhizospheric bacteria and whether this ability provides growth advantage to these organisms under iron-limited conditions.

A collection of 17 nodule isolates from C. cajan plants collected locally were used in this study. Only a few root nodule isolates tested produced both hydroxamate and catecholate type of siderophores, with the majority being similar to rhizobia from cowpea (Modi...
et al., 1985; Jadhav and Desai, 1992) and chick pea (Roy et al., 1994) in being able to produce only catecholate type of siderophores. Considerable variation was found in the amount of siderophore produced ranging from approximately 5–100 μg/ml. Rhizospheric bacteria from roots of locally collected pigeon pea and groundnut plants on the other hand were found to be both catecholate and hydroxamate producers. Powell et al. (1980) reported that soils contain significant amounts of hydroxamate type siderophores and Jurkevitch et al. (1992) have shown that a major proportion of rhizospheric and soil bacteria are able to utilize the Fe complexes of the hydroxamate siderophores as Fe source. Consistent with these observations is our finding that rhizospheric isolates predominantly produced more amounts of hydroxamate type siderophores with some isolates producing almost 300 μg/ml.

Carson et al. (1992) have reported variation among strains of rhizobia in terms of amount of iron required for optimal growth and their response to iron availability. The present work shows root nodule bacteria from the same plant species can vary in terms of quantity and quality of siderophore produced as well as the ability to cross-utilize heterologous siderophores. The root nodule bacteria tested here were proficient at cross-utilization of siderophores produced by not only other nodule isolates but also by rhizospheric isolates. Interestingly, even isolates from the nodules of the same plant species from a single field showed diverse patterns of siderophore cross-utilization, e.g. GAU(2)4b and GAU(2)5 which are isolates from two different nodules of the same plant did vary in their siderophore cross-utilization profile. The zone of growth around the siderophore containing well/disc on an iron-limited medium indicates either that the test strain can directly utilize the siderophore applied or that the siderophore synthesized by the test strain can exchange Fe³⁺ from the one that is applied. The cross-utilization results revealed that nodule isolates preferentially utilized homologous siderophores, which are mainly comprised of catecholate types over the heterologous ones, which are predominantly hydroxamate type. These results lead us to conclude that most of the nodule isolates lack receptors for hydroxamate type siderophores made by other rhizospheric species and that the catecholate type siderophore receptors produced by root nodule bacteria are perhaps comparatively weaker than their hydroxamate counterparts.

The increased growth of GHU(iii) in the presence of exogenously added mixture of homologous and heterologous siderophore (Figs. 2 and 3) could be attributed to presence of receptors for heterologous siderophores in that strain enabling the organisms to utilize these siderophore complexes as an iron source. The failure to show increase in growth of AP(6)4 in the presence of exogenous siderophore could be lack of respective receptors in that strain. The growth stimulation of GHU(iii) in co-inoculation studies with strain 88 can also be explained on the same basis. Strain 88 is one of the rhizospheric isolate known to produce only hydroxamate type of siderophore at higher concentration (185 μg/ml). Previous studies from this laboratory have shown induction of 76 and 80 kDa outer membrane protein in cowpea Rhizobium GN1 and these proteins have been shown to be the receptors for catecholate siderophores (Jadhav and Desai, 1994).

Out membrane protein profile of GHU(iii) and AP(6)4 showed induction of an approximately 80 kDa protein in both the strains under iron starved conditions but no significant difference could be observed between the high and low cross-utilizing strains. Thus, the high siderophore cross-utilizing ability of GHU(iii) cannot be attributed to the constitutive presence of multiple iron repressible receptors but it may be likely that GHU(iii) siderophore receptor has broad specificity as it can cross-utilize hydroxamate siderophores as well as desferal. E. coli FhuA, the receptor for ferrichrome has been shown to interact with rifamycin CGP4832 which is not structurally similar to ferrichrome (Ferguson et al., 2001). Although inducible siderophore receptor expression has not so far been demonstrated in rhizobia, this possibility cannot be ruled out. Further work on Fe-siderophore receptors of these strains would confirm this hypothesis.

5. Conclusion

It is concluded from the present study that C. cajan nodule isolates show preferential production as well as utilization of catecholate type siderophores over hydroxamate type. The isolate GHU(iii) showed growth stimulation in presence of exogenously added homologous as well as heterologous siderophores which it can cross-utilize and this has been further substantiated by co-inoculation studies. It also showed the ability to cross-utilize desferal (a hydroxamate type siderophore) as well as catecholate type siderophores whereas AP(6)4 and GAU(1)1 did not. Induction of an approximately 80 kDa outer membrane protein in both GHU(iii) and AP(6)4 under iron-limited condition was observed showing no significant difference in the siderophore receptor profiles of both the strains indicating that GHU(iii) may possess a broad specificity siderophore receptor.
Acknowledgement

The authors acknowledge the financial support of this project by Department of Biotechnology, Government of India, as a grant (No. BT/PR2420/AGR/21/118/2001) to A.J.D. and G.A.

References


Functional expression of *Escherichia coli fhuA* gene in *Rhizobium* spp. of *Cajanus cajan* provides growth advantage in presence of Fe\(^{3+}\): ferrichrome as iron source

Geetha Rajendran · Shreni Mistry · Anjana J. Desai · G. Archana

Abstract *Cajanus cajan* rhizobial isolates were found to be unable to utilize iron bound to ferrichrome, desferrioxamine B or rhodotorulic acid, all being hydroxamate type siderophores. A broad host range expression vector containing the *Escherichia coli fhuA* gene, encoding the outer membrane receptor for Fe-ferrichrome, was constructed. The plasmid construct (pGR1), designed to express *fhuA* under the *lac* promoter of *E. coli*, complemented *E. coli* MB97 ΔfhuA mutant for ferri-ferrichrome utilization and also allowed *Rhizobium* spp. ST1 and *Rhizobium* spp. IC3123 to grow using iron bound to ferrichrome. Sensitivity to the antibiotic albomycin, transported via the FhuA receptor, was found in case of MB97 as well as rhizobial transformants harboring pGR1. The rhizobial transformants expressing *fhuA* showed growth stimulation when co-inoculated with *Ustilago maydis*, a fungal species known to produce ferrichrome under iron starved conditions. Growth stimulation was also observed in the presence of externally supplied ferrichrome. The significance of these findings in terms of the potential for improving the survivability of rhizobial bioinoculant strains in natural soils is discussed.

Keywords *Rhizobium* spp. · Ferrichrome uptake · *E. coli fhuA* gene · Heterologous gene expression · *Cajanus cajan* · Siderophore utilization

Introduction

To meet iron requirements for growth, most microorganisms have developed high affinity iron uptake systems for scavenging and transporting ferric iron (Braun and Killmann 1999). These involve low molecular weight compounds called siderophores which bind ferrie iron with high affinity (Neilands 1995; Crosa and Walsh 2002) and several membrane bound and periplasmic proteins that function together in the uptake of the ferric-siderophore complex (Faraldo-Gómez and Sansom 2003). More than 500 distinct siderophores have been reported to be secreted by microorganisms and their iron ligation groups have been classified into three main chemical types: hydroxamate, catecholate and hydroxyacid (Wandersman and Delepelaire 2004).

Rhizobia are a group of gram-negative bacilli possessing the ability to form a nitrogen-fixing symbiosis with members of the *Leguminaceae* family of plants. Recent taxonomy recognizes major groups of rhizobia as belonging to the *Mesorhizobium-Sinorhizobium-Rhizobium* group, the *Bradyrhizobium* group, the *Azorhizobium* group, *Methyllobacterium* group, and *Burkholderia* group (Zakhia and deLajudie 2001; Sahgal and Johri 2003). While many rhizobial strains have the potential to increase plant growth and yields, poor nodulation efficiencies due to low survivability as free-living soil microorganisms is a problem and iron limitation is one of the important factors contributing to their low survivability (Hemantaranjan and Garg 1986; O’Hara et al. 1988). Diverse types of siderophores are produced by the different rhizobial genera. For instance, *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti* secrete hydroxamate siderophores (Carson et al. 2000; Persmark et al. 1993). Citrate is the siderophore...
secreted by *Bradyrhizobium japonicum* (Guerinot et al. 1990). Several uncharacterized catecholate siderophores are produced by rhizobia of chick pea (Roy et al. 1994), cowpea ( Modi et al. 1985; Jadhav and Desai 1992) and pigeon pea (*C. cajan*) (Khan et al. 2006).

In addition to being able to use their own ferri-siderophore complexes, *S. meliloti* and *B. japonicum* can also utilize iron complexed to siderophores made by other species (Smith and Neilands 1984; Plessner et al. 1993). Most notable among the heterologous siderophores utilized by these rhizobia is ferrichrome, a prototypical hydroxamate type siderophore secreted by several fungi such as *Ustilago* spp. (Payne 1994). The uptake system for Fe$^{3+}$-ferrichrome known as the *fhu* system in *Escherichia coli* (Braun 1995), consists of *fhuACDB* operon, of which *fhuA* encodes the multifunctional outer membrane protein (78 kDa) that acts not only as the ferrichrome-ion receptor but also as the receptor for several phages, for the bacterial toxin colicin M and for antibiotics such as albomycin (Ferguson et al. 2001).

Rhizobia shown to either produce or utilize hydroxamate types of siderophores have been found to possess genes similar to the *fhu* system of *E. coli* e.g. *R. leguminosarum* and *B. japonicum* have been shown to possess *fhuA* orthologs (LeVier et al. 1996; Yeoman et al. 2000) of which the *B. japonicum* gene *fegA* has been demonstrated to be responsible for the uptake of iron-ferrichrome complex (Benson et al. 2005). *S. meliloti* also has two *fhuA* homologues in its genome (Lynch et al. 2001) although no functional studies regarding them are available. Not much is however known about the hydroxamate uptake system in other rhizobia, especially those that synthesize catecholate types of siderophores. Earlier work from this laboratory has shown that majority of nodule isolates from *C. cajan* produce catecholate siderophores and that they are more proficient at utilization of heterologous catecholates rather than hydroxamates (Khan et al. 2006). In the present study we show that strains of rhizobia used as bioinoculants for *C. cajan* fail to utilize ferri-ferrichrome as well as iron bound to other hydroxamate siderophores. Expression of the *E. coli fhuA* gene enabled these bacteria to utilize ferri-ferrichrome as iron source and conferred upon them a better survival in presence of this siderophore.

**Materials and methods**

Bacterial strains, plasmids and growth conditions

Rhizobia IC3109, IC3123, IC3163 and IC3169 which are used as bioinoculants for *C. cajan* were obtained from Dr. A. K. Saxena, Indian Agricultural Research Institute (IARI), New Delhi, India. *Rhizobium* ST1 is a laboratory isolate obtained from *C. cajan* nodules. All nodule bacteria were routinely grown on Asby's Mannitol Agar (AMA) (Jadhav and Desai 1992). *U. maydis* MTCC 1474 and *Rhodotorula mucilaginosa* MTCC 850, obtained from microbial type culture collection (MTCC), Chandigarh, India, were used as the sources of ferrichrome and rhodotorulic acid, respectively (1994). Plasmid bearing strains of *E. coli* and rhizobia were maintained in Luria Bertani (LB) medium and Asby's Mannitol Broth (AMB), respectively with appropriate antibiotics added at concentrations as follows: ampicillin 100 µg/ml, chloramphenicol 34 µg/ml, co-trimethoprin 60 µg/ml. *E. coli* MB97A*fhuA* mutant, obtained as a kind gift from Prof. Volkmar Braun, Institute of Microbiology, University of Tuebingen, Germany was used for the phenotypic complementation. *Pseudomonas* strains were used as a source of heterologous siderophores. Table 1 depicts the microbial strains and plasmids used in the study.

Siderophore production and utilization tests

Chrome Azurol-S (CAS) plates were used to detect siderophore production (Schwyn and Neilands 1987). Rhi zobial cultures were grown in deferrated AMB for 48 h and centrifuged at 7,500g for 20 min. The culture supernatant was used to detect the type of siderophore produced and to quantify it. Catecholate type siderophores were estimated using 2,3-dihydroxybenzoic acid (2,3-DHBA) as a standard (Payne 1994). Hydroxamate siderophores were estimated by Gibson–Magrath method using hydroxyamine hydrochloride as a standard (Gibson and Magrath 1969). To check for siderophore utilization, AM plates containing EDTA at the respective minimum inhibitory concentration for each strain were surface spread with the test organism. Wells of 8 mm diameter were bored, FeSO$_4$ (100 µg/ml) and saline (pH 8.0) were added as positive and negative controls respectively. Ferrichrome (Sigma-Aldrich Co., USA) 50 µg/ml, Desferrioxamine B (Desferal, Novartis Basle, Switzerland) and ferric citrate was added at 100 µg/ml into the wells. Wherever siderophore produced by microbial strains was to be used, 100 µl of filter sterilized culture supernatant grown under iron limited conditions was added into the wells. Plates were incubated at 30 ± 2°C and growth around wells (zone of growth exhibition) was monitored after 48 h.

Identification of the rhizobial strains

A fragment corresponding to 1,100 bp of the 16S rRNA gene was amplified using the universal eubacterial
Table 1 Microbial strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Bacterial/fungal strains or plasmids</th>
<th>Properties*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Used for plasmid transformation</td>
<td>Sambrook and Russell (2001)</td>
</tr>
<tr>
<td>S17-1</td>
<td>Used for conjugal transfer of pGR1 into rhizobia</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>BL21(DE3)::pET17b/fhuA</td>
<td>Used as source of the <em>fhuA</em> gene</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>MB97</td>
<td>AB2847 Δ<em>fhuA</em></td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Rhizobial isolates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC3109</td>
<td>Wild type, bioinoculant used for <em>C. cajan</em></td>
<td>IARI, New Delhi, India</td>
</tr>
<tr>
<td>IC3123</td>
<td>Wild type* bioinoculant used for <em>C. cajan</em></td>
<td>IARI, New Delhi, India</td>
</tr>
<tr>
<td>IC3163</td>
<td>Wild type bioinoculant used for <em>C. cajan</em></td>
<td>IARI, New Delhi, India</td>
</tr>
<tr>
<td>IC3169</td>
<td>Wild type*</td>
<td>IARI, New Delhi, India</td>
</tr>
<tr>
<td>ST1</td>
<td></td>
<td>Lab. Isolate</td>
</tr>
<tr>
<td><strong>Pseudomonas strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> KT2440</td>
<td>Used as a source of heterologous siderophores</td>
<td>Lab. collection</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ATCC1525</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> MTCC2453</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ustilago maydis</em> 1474</td>
<td>Used as a source of ferrichrome</td>
<td>MTCC, Chandigarh, India</td>
</tr>
<tr>
<td><em>Rhodotorula mucilaginosa</em> 850</td>
<td>Used as a source of rhodotorulic acid</td>
<td>MTCC, Chandigarh, India</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTUC203</td>
<td>pACYC184 mejABCD, Cm⁠</td>
<td>Solbiati et al. (1996)</td>
</tr>
<tr>
<td>pGR1</td>
<td>pUCPM18 with fhuA, Ap⁠</td>
<td>This study</td>
</tr>
</tbody>
</table>

*mob* mobilization

* Ap⁠ and Cm⁠ are ampicillin and chloramphenicol resistance, respectively

* Rhizobial isolates used for transformation with pGR1

** primers 5'-GAGAGTTTGATCCTGGCTCAG-3' (forward primer) and 5'-GCTCGTTGCGGGACCTT AAACC-3' (reverse primer). Approximately 800 bp sequence information of the amplified fragment was obtained through the sequencing service provided by Bangalore Genei Pvt. Ltd., India. The sequence data were matched using tools provided at Ribosomal Database Project (RDP) II.

**Construction of the vector pGR1**

Plasmid pET17b containing *E. coli* fhuA gene (gifted by Dr. Ranjan Chakraborty, East Tennessee State University, USA) was digested with *BglII/HindIII* and ligated to *BamHI/HindIII*-digested pUCPM18 (Hester et al. 2000), resulting in pGR1 in which the expression of fhuA is driven under the *lac* promoter of *E. coli*. pGR1 was transformed into rhizobial bioinoculants strains by either electroporation (12.5 kV/cm) using Eppendorf electroporator 2510 or by conjugation with *E. coli* S17.1 strain carrying pGR1. Standard techniques were used for isolation of plasmids, restriction digestion, ligation, agarose gel electrophoresis and transformation (Sambrook and Russell 2001).

**Functional expression of fhuA gene of pGR1**

*Escherichia coli* MB97 was transformed with pGR1 and the transformants checked for ferrichrome utilization, albomycin sensitivity and sensitivity to colicin M and microcin J25. Albomycin samples as well as *E. coli* strains containing pTO4 cma cmi and pTUC203 mejABCD were kindly gifted by Prof. Volkmar Braun, Institute of Microbiology, University of Tuebingen, Germany. Supernatants of *E. coli* strains containing pTO4 cma cmi and pTUC203 mejABCD were used directly as the sources of colicin M and microcin J25, respectively. Expression of fhuA gene in *E. coli* MB97 as well as rhizobial transformants was checked using ferrichrome (50 μg/ml) utilization test as described above.

**Growth assays and co-inoculation studies**

The growth of the parental and rhizobial transformant strains was compared in presence and absence of pure ferrichrome (15 μM). Growth experiments were also performed in presence and absence of *U. maydis* under iron-starved conditions. Freshly grown rhizobial
cultures (10^2 cfu/ml) were co-inoculated with freshly grown U. maydis (10^2 cfu/ml) in nutrient broth supplemented with 1 mM EDTA for creating iron-limited conditions. Siderophore production was checked by CAS assay solution in aliquots withdrawn at different time intervals. Growth of bacteria and fungus was monitored on nutrient agar plates by counting the colony forming units (cfu/ml) at regular intervals.

Outer membrane protein (OMP) profiling

Outer membrane extraction was performed as per Guan et al. (2001). E. coli strains MB97 and MB97::pGR1 were grown in LB till A600 of 0.6 and were induced with IPTG (0.4 mM) for 2 h. Rhizobial transformants and parent strains were grown in AMB for performing the OMP extraction. The protein profiles of parent strains were compared with those of the transformants on SDS-PAGE to identify FhuA expression.

Results

The studies on siderophore production and utilization of heterologous siderophores by C. cajan rhizobial bioinoculants showed that most of the strains failed to utilize iron bound to desferrioxamine B, ferrichrome or rhodotorulic acid produced by R. mucilaginosa MTCC 850 (Table 2). Many of the rhizobial strains were able to utilize the siderophores produced by P. putida KT2440 (producer of pseudobactin and pyoverdine like siderophores), but were unable to cross-utilize ferrichrome, rhodotorulic acid and desferrioxamine B (siderophores produced by fungi and actinomycetes, respectively) (Table 2). Rhizobial strains IC3123 and ST1 were selected for transformation with E. coli fhuA containing plasmid pGR1. 16S rRNA gene partial sequence of isolates IC3123 (Genbank Accession number DQ632607) and ST1 (Genbank Accession number DQ632608) showed them to bear 95 and 96.7% sequence similarity with Rhizobium spp., respectively.

The plasmid pGR1 was constructed by ligating a 2.3 kb fragment containing the fhuA gene of E. coli into pUCPM18 vector so as to allow fhuA expression under the control of the lac promoter of E. coli available in the vector. The construct pGR1 was checked by RE digestion (data not shown) using XbaI/HindIII, which showed expected band pattern. The expression of the pGR1 in E. coli MB97 (fhuA deficient mutant) was confirmed by phenotypic complementation (Fig. 1b). As compared to the parental strain, the MB97::pGR1 transformant strain was sensitive to the antibiotic albomycin and to microcin J25 and colicin M (all of which are transported through the FhuA receptor). The fhuA gene expression in MB97 strain was further confirmed by specific induction of a 78 kDa outer membrane protein in MB97::pGR1 under IPTG induced conditions which was absent in the parental strain (Fig. 1a).

The plasmid pGR1 was transformed into rhizobial strains IC3123 and ST1 and the transformants obtained could utilize ferri-ferrichrome (Fig. 2a) and a zone of inhibition was obtained in presence of albomycin (Fig. 2b) indicating sensitivity to this antibiotic. The outer membrane preparation of IC3123::pGR1 and ST1::pGR1 showed the induction of a protein band of approximately 78 kDa that was absent in the corresponding parental strains (Fig. 3). IC3123::pGR1 (Fig. 4b) and ST1::pGR1 (data not shown) showed growth stimulation when co-inoculated with U. maydis whereas the parent strain failed to show such growth stimulation (Fig. 4a). Addition of ferrichrome (15 μM) to the growth medium, stimulated growth of transformant strains and not the parental strains (Fig. 4c, d).

Discussion

The rhizosphere as an ecosystem consists of microbial populations engaged in competitive interactions for limiting nutrients, iron being one of them (Somers and Srinivasan 2004). Interest in the iron uptake mechanisms of root nodule bacteria emerges from the understanding that iron-containing proteins are prominent members of the nitrogen-fixing machinery (Guerinot 1991). Rhizobial bioinoculant strains used in the present investigation were found to produce and utilize catecholate type siderophores but were poor producers as well as utilization of hydroxamate-type siderophores produced by fungi and actinomycetes.

Table 2 Siderophore production and utilization by C. cajan rhizobial strains

<table>
<thead>
<tr>
<th>Rhizobial strains</th>
<th>Siderophore production (μg/ml)</th>
<th>Siderophore utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe-cit</td>
<td>Desf</td>
</tr>
<tr>
<td>IC3109</td>
<td>1.342 (C)</td>
<td>+</td>
</tr>
<tr>
<td>IC3123</td>
<td>2.376 (C)</td>
<td>+</td>
</tr>
<tr>
<td>IC3163</td>
<td>2.376 (C)</td>
<td>+</td>
</tr>
<tr>
<td>IC3169</td>
<td>2.046 (C)</td>
<td>+</td>
</tr>
<tr>
<td>ST1</td>
<td>16.5 (C)</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates growth and — no growth around the siderophore containing wells

Fig. 1  a Outer membrane protein profiles of *E. coli* MB97::pGRl (lane 2) and *E. coli* MB97 (lane 3) when induced with 0.4 mM IPTG. Arrow head indicates band of approximately 78 kDa specifically induced in plasmid containing strain. b Effect of addition of a colicin M; b microcin J25 and c albomycin on *E. coli* MB97 (left panel) and on *E. coli* MB97::pGRl (right panel). Zones of inhibition observed in case of the transformant indicate sensitivity to all the antimicrobial compounds and thereby confirm functional expression of *fhuA*

Fig. 2  a Ferrichrome (50 μg/ml) utilization by rhizobial transformants. Parent strains ST1 and IC3123 do not show zone of exhibition whereas the transformants ST1::pGRl and IC3123::pGRl of siderophores. A similar observation was made earlier by our group with native *C. cajan* root nodule isolates obtained from local fields (Khan et al. 2006). The inability to utilize hydroxamates could be considered as a negative fitness factor since hydroxamate siderophores are found in significant amounts in natural soils (Powell et al. 1980). Ferrichrome, a fungal siderophore, is found in nanomolar concentrations as estimated by physicochemical (Holmstrom et al. 2004) as well as bioassay methods (Powell et al. 1983). As majority of soil bacteria are good utilizers of iron bound to hydroxamates (Jurkevitch et al. 1992), the rhizobia studied here would be at a competitive disadvantage when residing free in soils.

The present work was aimed at understanding the possible impact of ferrichrome utilization in rhizobial growth and survivability under conditions wherein ferrichrome is made available by other producer species. To achieve this objective, the *E. coli* *fhuA* gene was heterologously expressed in *C. cajan* rhizobia. Since the *lac* promoter provides a good constitutive expression system in rhizobia (Labes et al. 1990) the
That the expression of the outer membrane receptor alone enabled the rhizobia to take up ferrichrome implies that additional transport activities involving periplasmic and inner-membrane bound proteins are present in the strains and are perhaps part of uptake machinery for some other unknown siderophore but are being recruited for ferrichrome utilization. A similar finding has been reported Brickman and Armstrong (1999) in a study dealing with the fauA gene encoding the receptor for alcaligin, a siderophore produced by Bordetella species. The authors found that the incorporation of fauA gene alone could confer upon a siderophore deficient strain of P. aeruginosa the ability to utilize ferric alcaligin. These observations reinaugurate the belief that among all the ligand-protein interactions of members of the bacterial iron-acquisition system, the binding of ferri-siderophores to the outer membrane receptor proteins is the most specific (Guérinot 1994). For instance, in E. coli separate outer membrane receptors transport ferric iron bound to aerobactin, ferrichrome, rhodotorulic acid and ferrirhoxamine, yet all use a common set of periplasmic and inner membrane components. Genome sequences of bacteria contain numerous putative ferri-siderophore receptor genes (Cornelis and Matthijs 2002; Poole and McKay 2003) but do not contain equivalent copies of the genes for periplasmic and cytoplasmic membrane-bound proteins. Our results provide evidence that engineering rhizobial strains by incorporating genes for multiple iron-siderophore receptors has potential...
in increasing the suite of ferric-siderophores that they can utilize.

The growth stimulation of only rhizobial transformant IC3123::pGR1 and not the parent IC3123 was observed when pure ferrichrome (15 μM) was exogenously supplied. This substantiates that the rhizobial transformants are at a competitive advantage relative to the untransformed strains under conditions when iron is available only as bound to ferrichrome. Similar observation was made when ferrichrome-producing organism _U. maydis_ was co-inoculated with the rhizobia. When pure ferrichrome was supplied externally it was observed that the growth rate of rhizobial transformants was somewhat lower than the parent strain. This may be attributed to metabolic load imposed by the presence of plasmid since it is well-documented that expression of plasmid encoded marker genes or inserted foreign genes impose metabolic burden on the organism (Glick 1995). Comparison of the growth rates of rhizobia bearing the vector plasmid and those harboring pGR1 would help discern whether the growth disadvantage is due to marker gene expression or _fhuA_ expression. It is puzzling why a similar observation was not seen when the rhizobial transformants were co-inoculated with _U. maydis_. It may be speculated that this could be due to the production of some factors by _U. maydis_ that helped the rhizobia to retain their normal growth profile.


Acknowledgments The financial support of this project by Department of Biotechnology, Government of India, as a research grant (no: BT/PR2420/AGR/21/118/2001) to A.J.D. and G.A. is acknowledged.

References


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