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Mutants of *Azospirillum brasilense* Altered in the Uptake of Iron

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Abstract

The root associated, nitrogen fixing bacterium *Azospirillum brasilense* produces catechol type siderophores. Cells of *A. brasilense* strain SPF94 have been mutagenized with transposon Tn5 and mutants have been isolated that were impaired in the ability to take up iron from the medium. A preliminary characterization of these mutants including: growth capability in iron-limiting medium, excretion of catechol compounds that are presumed to be the siderophores of the species, and uptake of ^{55}Fe to the cells is reported.

Keywords: *Azospirillum*, siderophores, mutants

1. Introduction

Iron is the fourth most abundant element in the Earth's crust, however under aerobic conditions and at neutral pH it is present as a component of insoluble minerals (Neilands, 1981a). Bacterial growth, on the other hand, depends on the availability of iron, an essential nutrient that participates in many biological processes and is a cofactor of several enzymes including, for nitrogen fixing bacteria, the nitrogenase proteins (Carnahan and Castle, 1958). Therefore, the possession of specialized iron transport systems may be crucial for bacteria to override the iron limitation imposed by the environment.

Siderophores are low molecular weight compounds produced by microorganisms, able to bind iron from the environment. The binding to the siderophore allows transfer of iron to the cell, enabling bacteria to compete for this otherwise unavailable element (Neilands, 1981b).

Studies with some rhizosphere bacteria that promote plant growth showed that the siderophore mediated iron uptake systems of these microorganisms exert a strong influence on the microbial community that can be quite beneficial to the plant (Kloepper et al., 1980; Schroth and Hancock, 1982).

Nitrogen fixing bacteria of the genus *Azospirillum*, which live associated with the roots of grasses and other plants utilized in agriculture, also produce siderophores that represent an important factor for their competition and survival in the rhizosphere (Hartmann, 1989). The siderophore *Spirilobactin*, produced by *A. brasilense* strain RG, is a catechol containing 2–3 dihydroxybenzoic acids ornithine and serine (Bachhawat and Gosh, 1987a). *A. lipoferum* strain D-2 also produces catechol type siderophores (Saxena et al., 1986), one of them is also involved in the transport of molybdenum (Saxena et al., 1989). Although some information has recently been obtained on the control of siderophore synthesis (Bachhawat and Ghosh, 1989) and on the expression of iron transport outer membrane proteins (Bachhawat and Ghosh, 1987b), nothing is known on the genetics of siderophores in *Azospirillum*. In the present communication we describe the isolation and partial characterization of mutants of *A. brasilense* strain SPF94, impaired in the ability to take up iron from the medium.

2. Materials and Methods

Bacterial strains and plasmids

Azospirillum brasilense strain SPF94 *rif* (Fani et al., 1988) and *Escherichia coli* strain DH1 (Hanahan, 1983) were used. Plasmid pGS9 (Selvaraj and Iyer, 1983) was used as donor of transposon Tn5.

Growth conditions

E. coli and *A. brasilense* were routinely grown on Py (Bacto Antibiotic Medium n.3, Difco); iron starvation conditions for *A. brasilense* were obtained in TM medium (in one liter of Tris buffer 50 mM, pH 7.5, KH_2PO_4 0.3 g; NaCl 0.5 g; NH_4Cl 1 g; $CaCl_2$ 10 mg; $MgSO_4 \cdot 7H_2O$ 200 mg; fructose 2 g and yeast extract 40 mg were added). TM medium was supplemented with $FeCl_3$ or with the iron chelator 2-2′-dipyridyl as indicated in the results section. Siderophore production by *Azospirillum* colonies was detected on CAS (chrome azurol-S)

plates or on the supernatant of cultures with the CAS reagent prepared as described by Schwyn and Neilands (1987). Growth was at 33°C.

Conjugation

Conjugation between *E. coli* DH1 carrying plasmid pGS9 and recipient *A. brasilense* was carried out as described by Singh and Klingmuller (1986).

^{55}Fe uptake

A. brasilense cells were grown on TM without iron, with and without 2–2′-dipyridyl, up to about $0.D._{590} = 0.5$; to 3 ml aliquots of the culture, 50 μl of $^{55}FeCl_3$ (0.2 mM, 0.02 μCi/μl, Amersham) were added. Samples (0.5 ml) were removed at timed intervals, filtered through polycarbonate membranes (0.6 μm pore size, Millipore) that were then washed with 3 ml of TM without iron. Radioactivity on dried filters was measured on ^{14}C channel of a Kontron scintillation counter. Zero time samples were obtained by adding ^{55}Fe to ice cold cultures that were filtered as described before; counts of the zero time samples were subtracted from counts of the other samples.

Catechol determination

Catechol in 1 ml samples of bacterial cultures supernatant was determined by the Arnow test (Arnow, 1937) using 3,4-dihydroxyphenylalanine (DOPA) as standard.

DNA manipulation

Any DNA manipulation and Southern blotting was carried out according to Maniatis et al. (1989).

3. Results and Discussion

Isolation of mutants

A. brasilense strain SPF94 cells were mutagenized with transposon Tn5 using the plasmid pGS9. About 10^4 kanamycin resistant transconjugants were isolated and checked for the production of the orange halo around the colonies on the blue CAS plates. Five putative mutants, referred to as S1, S2, S4, S5 and S6, were selected for their inability to form detectable halos on CAS plates after 3 days of incubation, when colonies of the parental strain produced conspicuous halos. Mutant S6 grew poorly on the CAS plates and did not show

any halo even after 8 days of incubation. The other mutants grew well on the CAS plates and started to produce a visible halo around the colonies after 6–8 days of incubation.

The total DNA of the five mutants was purified, double digested with EcoRI, which does not cut inside the sequence of Tn5, and BamHI, which cuts once inside the transposon, electrophoresed, blotted on a membrane and hybridized with labelled Tn5 DNA.

Results, reported in Fig. 1, indicated that the transposon was inserted on

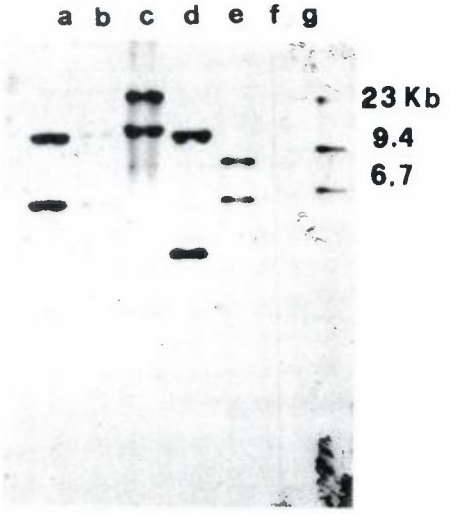

Figure 1. Southern blot of total DNA of *Azospirillum* strains digested with EcoRI and BamHI and hybridized with Tn5 probe. Lines: a = S1, b = S2, c = S4, d = S5, e = S6, f = SPF94, g = marker.

mutants DNA inside fragments of different size and therefore affected different genes, with the exception of mutants S1 and S2 that showed the same hybridization pattern and are probably to be considered as carrying the same mutation.

Characterization of mutants

The ability of the selected mutants to scavenge iron from the medium was compared to that of SPF94 strain by growth experiments in liquid medium. Figure 2 shows growth curves of the five mutants and the parental strain in media with and without iron and with the addition of the iron chelator 2-2′-dipyridyl. Results indicated that the growth of all the strains was impaired by the lack of iron, however, the mutants appeared more sensitive to iron deprivation than the parental strain, particularly the growth of mutants S5

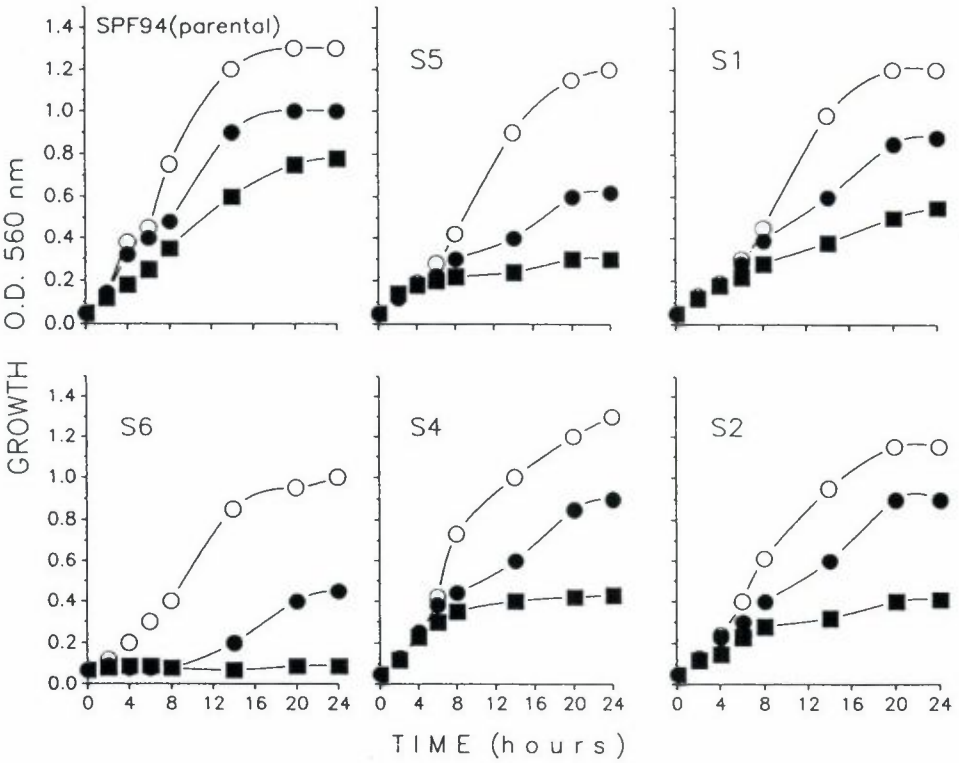

Figure 2. Growth curves of *A. brasilense* parental and mutant strains on TM medium with iron (10 μM), open circles; without iron, filled circles; and with 2-2′-dipyridyl (60 μM), filled squares. Cells were grown in Py, washed in TM, diluted in the appropriate medium to $O.D._{590} = 0.05$ and grown at 33°C with shaking.

and S6 was almost completely inhibited in the presence of the iron chelator. The addition of iron during the growth in iron limiting media, restored the growth rate of all the strains (not shown).

Catechol excretion

Siderophores of *A. brasilense* have been reported to belong to the catechol type (Bachhawat and Gosh, 1987a); for this reason we tested the ability of the selected mutants, growing in iron-free medium, to excrete catechol compounds. Results reported in Fig. 3, indicated that the mutant and the parental strains excreted low levels of catechol from the beginning to the end of the growth, without remarkable differences among the strains, with the possible exception of mutant S5 which excreted a reduced level of catechol. Moreover, results indicated that the addition of 2-2′-dipyridyl did not induce higher catechol

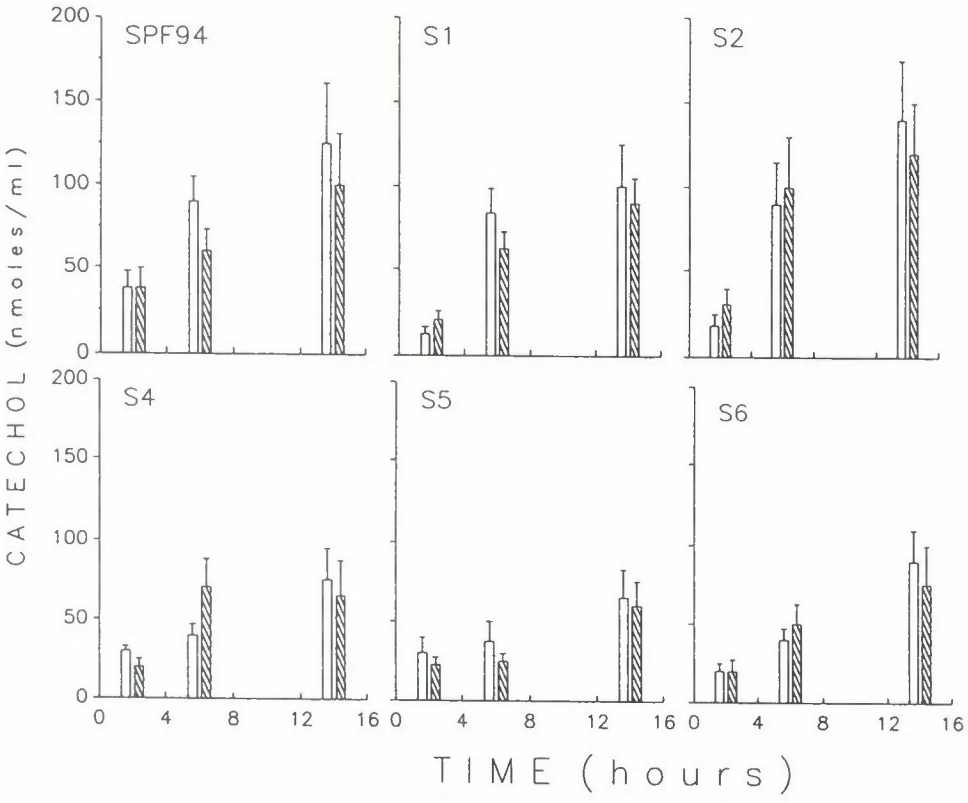

Figure 3. Catechol excretion by *A. brasilense* parental and mutant strains. Cells were grown as described in Fig. 2, without iron (blank bars); and with 60 μM 2-2′-dipyridyl (diagonal bars); at indicated time samples were withdrawn and catechol content assayed. Data are means of four replicates in two experiments.

excretion. Further studies will be necessary to clarify whether and to what extent these catechol compounds measured with the Arnow test represent the siderophore produced by *A. brasilense* strains. We in fact tested the excretion of siderophores in the supernatant of *Azospirillum* cultures also by the CAS assay; results, not shown, indicated that only the parental strain was able to produce detectable amounts of CAS positive compounds (siderophores), confirming the phenotype of the mutants observed on the CAS plates.

Iron uptake

As the mutants did not seem impaired in catechol production, we tried to investigate whether the observed phenotypes were related to the inability to

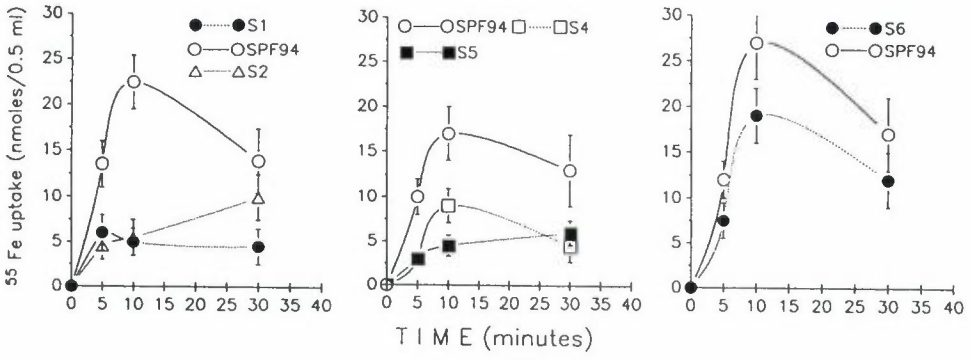

Figure 4. ^{55}Fe uptake to cells of *A. brasilense* parental and mutant strains. Experiments were carried out as described in Methods; data are means of three replicates.

take up iron from the medium. Figure 4 reports the results of ^{55}Fe-uptake by cultures of *A. brasilense* strains. Data obtained indicated that mutants S1, S2, S4 and S5 had a reduced rate of iron uptake compared to that of the parental strain, while mutant S6 showed a take up of iron at the same level as the parental strain. Growing the cells in medium containing 2-2′-dipyridyl did not affect iron uptake of the parental strain and the mutants (not shown).

Although we cannot at present draw solid conclusions about the functions presumably altered in the mutants described, some tentative working hypothesis could be put forward. Mutants S1 and S2 which appear identical by genetic evidence, showed a phenotype similar to that of strains S4 and S5; they have a reduced ability to take up iron that correlates with reduced growth on severly iron-limiting medium (addition of 2-2′-dipyridyl). These mutants produce a catechol compound that probably is not the true siderophore, suggesting that the mutation could affect the siderophore biosynthesis. Mutant S6 takes up iron at the parental strain level, however, showed a more drastic phenotype in respect to halo production and growth in iron-limiting medium. A possible explanation is that this strain was unable to utilize the iron taken up by an otherwise unaffected uptake system.

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