Iron Transport in *Azospirillum brasilense*: Role of the Siderophore Spirilobactin

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A catechol-type siderophore was secreted by *Azospirillum brasilense* in the growth medium when the cells became iron-deficient. The siderophore, which we call spirilobactin, was purified and a partial characterization of its structure by hydrolysis revealed that it contained 2,3-dihydroxybenzoic acid, ornithine and serine in equimolar amounts. A high-affinity iron transport system capable of ^{59}Fe uptake from a $^{59}\text{Fe(III)}$ –spirilobactin complex was also induced in *A. brasilense* grown under iron deficiency. Kinetic studies on the iron uptake system revealed that it was carrier-mediated, with K_m and $V_{\max}$ values of $0.23\ \mu\text{M}$ and $0.27\ \text{nmol Fe min}^{-1} (\text{mg cell protein})^{-1}$, respectively. Dependence of iron uptake on an energized membrane, its insensitivity to arsenate and inhibition by protonophores suggest that transport is driven by the proton gradient across the membrane. Iron-deficient *Azospirillum lipoferum* transported ^{59}Fe from the $^{59}\text{Fe(III)}$ –spirilobactin complex (at a rate 3-fold lower than that of *A. brasilense*) but *A. amazonense* failed to show uptake of iron under the same conditions.

INTRODUCTION

Iron is required for growth in trace amounts by all micro-organisms with the sole possible exception of the lactobacilli (Neilands, 1981). Although it is an abundant element in the earth's crust, the extreme insolubility of iron at physiological pH values under an aerobic atmosphere creates a situation where the soluble iron concentration falls far below that required for optimal microbial growth. Micro-organisms have therefore evolved a capacity to generate specific iron-chelating compounds or siderophores which can form tight, soluble complexes with iron, and also specific receptor proteins, present in the outer membrane of Gram-negative bacteria, that recognize these complexes and facilitate iron transport into the cells (Neilands, 1982). The specificity and high affinity for iron of these transport systems enable micro-organisms to compete with other organisms for the available iron; in the case of pathogenic bacteria this involves competing with the hosts' iron-binding proteins (Weinberg, 1984; Brown & Williams, 1985; Neilands & Leong, 1986). Studies with some rhizosphere bacteria that promote plant growth have shown that the high-specificity, high-affinity iron uptake systems of these micro-organisms exert a strong influence on the microbial environment in the rhizosphere that can be quite beneficial for the plants (Kloepper *et al.*, 1980; Scher & Baker, 1982; Schroth & Hancock, 1982). Furthermore, in some cases plants also obtain their iron through iron–siderophore complexes (Cline *et al.*, 1984), and evidence for a specific uptake system for iron phytosiderophores in the roots of some grasses has recently been presented (Romheld & Marschner, 1986).

Abbreviations: LBS, Luria broth–succinate; SMM, succinate minimal medium; FMM, fructose minimal medium; EDDA, ethylenediamine-di(*o*-hydroxyphenylacetic acid); NTA, nitrilotriacetate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; *p*CMB, *p*-chloromercurobenzoic acid; DHBA, 2,3-dihydroxybenzoic acid.

Azospirillum brasilense is a Gram-negative, nitrogen-fixing bacterium that normally associates with the roots of certain plants of the grass family and can enhance productivity of the plant (Van Berkum & Bohlool, 1980). However, the factors responsible for this plant-growth-promoting activity, still remain unclear (Elmerich, 1984). Should *A. brasilense* show an ability to secrete a siderophore, it is quite conceivable that the bacterium might act like the plant-growth-promoting rhizobacteria through the antagonistic effect of the siderophore on plant pathogens (Buyer & Leong, 1986).

In an attempt to understand the iron uptake mechanisms in *A. brasilense*, we have investigated siderophore production induced by iron deficiency. We report here identification of a siderophore, which we call spirilobactin, secreted by *A. brasilense*, and our initial studies on the kinetic and some other characteristic properties of a high-affinity iron transport system of the bacterium which is mediated by this siderophore.

METHODS

Bacterial strains and growth media. The major part of this study was done with *A. brasilense* RG whose source and characteristics were described by Maulik & Ghosh (1986). *A. lipoferum* 59b and *A. amazonense* Y1 were obtained from N. R. Krieg (Virginia Polytechnic Institute, USA).

Azospirillum brasilense RG was grown in Luria broth (Miller, 1972)–succinate (LBS) or in succinate minimal medium (SMM) as described in the accompanying paper (Bachhawat & Ghosh, 1987), or in fructose minimal medium (FMM) which was the same as SMM except that 0.4% (w/v) fructose replaced sodium succinate. Iron was added as required from a freshly prepared membrane-filtered solution of ferric chloride. *A. lipoferum* 59b was grown in SMM containing 0.1% (w/v) yeast extract and *A. amazonense* Y1 was grown in Luria broth supplemented with 0.5% (w/v) sucrose, pH 6.0. Bacteria were grown at 32 °C in a shaker-incubator (100 r.p.m.).

Chemicals. Bacto-Tryptone and Bacto-Yeast Extract were obtained from Difco. Fructose and sodium succinate were obtained from Loba Chemical Co., India. Ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA), nitrilotriacetate (NTA), 2,3-dihydroxybenzoic acid (DHBA) and 3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine (ferrozine) were obtained from Sigma. Silica gel G was obtained from Merck and Tris was obtained from Fluka. Radioactive ^{59}Fe [760 mCi (g FeCl_3) $^{-1}$; 28.1 GBq (g FeCl_3) $^{-1}$] was supplied as ferric chloride in hydrochloric acid by Bhabha Atomic Research Centre, Bombay, India. All other reagents used were of analytical grade.

Assays and estimations. Protein was estimated by the dye-binding method of Bradford (1976). Protein in whole cells was assayed by a modification of the Lowry procedure (Herbert *et al.*, 1971). Bovine serum albumin was used as the standard for all protein assays. Iron was estimated by the ferrozine colorimetric method (Carter, 1971) except that the protein precipitation step was unnecessary in this case and was therefore omitted. Polypropylene tubes and tips were used for these assays. Ferrous ammonium sulphate was used as the standard for iron estimations.

Siderophores were estimated in terms of molar equivalents of DHBA by the Arnow method (Arnow, 1937) using DHBA as the standard. For the determination of the presence of hydroxamate compounds we used the method described by Atkin *et al.* (1970). Amino acids were estimated by the ninhydrin reaction according to the method described by Hirs (1967).

Thin-layer and paper chromatography. Thin-layer plates were prepared using silica gel G. Paper chromatography was done on Whatman 1 MM paper. The following solvent systems were used: (A) *n*-butanol/acetic acid/water (60:15:25, by vol.); (B) phenol/water (80:20, v/v); (C) *n*-butanol/pyridine/water (6:4:3, by vol.); and (D) 5% ammonium formate in 0.5% (v/v) formic acid.

Siderophores and amino acids were detected on chromatograms with 1% (w/v) ferric chloride and 0.2% ninhydrin in acetone respectively as spray reagents. Siderophores or catechols were also detected on chromatograms by UV illumination.

Isolation of outer membrane proteins. This was done by the Sarkosyl extraction procedure. SDS-PAGE of these preparations was done as described in the accompanying paper (Bachhawat & Ghosh, 1987).

Transport experiments. (i) *Induction of the siderophore-mediated iron uptake system in A. brasilense.* *A. brasilense* RG cells were grown overnight in SMM and reinoculated into the same medium without FeCl_3 . After 2 h growth, EDDA was added to a concentration of $10\text{ }\mu\text{g ml}^{-1}$. The cells continued to grow for about 4 h after which they entered stationary phase. At the beginning of stationary phase, cells were collected by centrifugation and washed with 0.5 vol. uptake buffer (see below). They were then resuspended in the same buffer at 32 °C to an OD_{590} of 0.8–1.0, and used immediately.

(ii) *Preparation of uptake buffer.* The uptake buffer (50 mM-potassium phosphate, pH 7.0, 0.2 mM-NTA, 0.025% sodium succinate) was made iron-free by the 8-hydroxyquinoline extraction method described by Rosenberg

(1979). Uptake buffer at other pH values was made using the same concentration of phosphate but adjusting the pH value by addition of KOH solution. The glassware used was soaked in chromic acid for a few hours, washed thoroughly and finally rinsed with double-distilled water. The uptake experiments were done in polypropylene tubes and all additions and withdrawals of radioactive iron were done with polypropylene tips.

(iii) *Preparation of the $^{59}\text{Fe(III)}$ -spirilobactin complex.* This was done by adding $^{59}\text{FeCl}_3$ to a 10-fold excess of spirilobactin (based on molar concentration of DHBA content) and then diluting with the uptake buffer to make the final concentration of Fe(III) equal to $10\ \mu\text{M}$. This mixture was then incubated at 32°C for 90 min with gentle shaking to allow for temperature equilibration and stable complex formation.

(iv) *Uptake assay procedure.* Washed cells (2 ml) were added to an equal volume of ^{59}Fe -spirilobactin solution maintained at 32°C in a shaker-incubator. Portions (0.5 ml) were withdrawn from the uptake mixture at intervals, filtered through membrane filters ($0.45\ \mu\text{m}$; Millipore), washed with 50 mM-potassium phosphate buffer, pH 6.8, containing 0.4 mM-NTA, and then dried and counted in a gamma counter (Beckman Gamma 5500). Blanks were prepared for each experiment by the same procedure except that cells were omitted and the volumes adjusted with uptake buffer.

RESULTS

Growth and siderophore production

The growth of *A. brasilense* RG under iron-limiting conditions was studied by exposing the organism to a range of iron concentrations. The growth curves of two of these cultures are shown in Fig. 1. The initial growth rate of all cultures was identical. However, the final amount of growth was greater with higher concentrations of iron in the cultures and was the least with no added iron. The absence of any striking difference between the ultimate growth in the low-iron- and high-iron-containing cultures is probably due to the residual iron present in the growth media (estimated to be $1.85\ \mu\text{M}$ -iron in the cultures with no added iron). In addition, the pH value of the medium was kept at 7.0 and fructose was the only carbon source, which helped to keep the iron in solution at neutral pH because of its mild iron-chelating activity (Charnley *et al.*, 1963). Fructose was used as the carbon source for these experiments instead of sodium succinate as with the latter the pH of the medium increased considerably during bacterial growth. Growth in cultures containing sodium succinate was much reduced even at higher iron concentrations and led to difficulties in correlating results.

To determine whether *A. brasilense* RG produced siderophores during iron limitation catechol- and hydroxamate-type compounds were assayed in the supernates of the iron-limited cultures. Only catechol-type compounds could be identified and their maximum production was achieved with 3–4 μM added iron (Fig. 1, inset). The ability of these compounds to function as siderophores was confirmed by the ability of a purified preparation of the catechol compound to reverse the effect of EDDA-induced growth inhibition of *A. brasilense* RG (Fig. 2) as effectively as the addition of FeCl_3 .

Siderophore purification

An overnight culture of *A. brasilense* RG in complete medium was diluted 100-fold with 4 l FMM containing 3–4 μM added FeCl_3 . The cultures were incubated with shaking for 22–30 h at 32°C until maximum siderophore production was achieved (equivalent to 200 nmol DHBA ml^{-1}). Cells were removed by centrifugation; the supernate was then acidified to pH 4.0 and kept in the dark at 4°C . Activated charcoal was added ($1.5\ \text{g l}^{-1}$), and after several hours of intermittent shaking the activated charcoal was collected by centrifugation, washed with 100 ml double-distilled water and the siderophore extracted with 200–300 ml 0.01% NH_4OH in 50% (v/v) ethanol. The extract was concentrated on a rotary evaporator to 5–10 ml, and then treated several times with an equal volume of chloroform. The aqueous phase was concentrated *in vacuo* at room temperature, the residue resuspended in the minimum amount of water and loaded onto preparative silica gel G plates. TLC was done with solvent system A. After chromatography, the plates were dried *in vacuo*, the bands detected by UV fluorescence (the siderophore gave a bright blue fluorescence), silica gel G scraped off and the siderophore extracted with 60% (v/v) ethanol. The siderophore was concentrated and traces of silica removed by membrane filtration. For analytical studies, the product so obtained was further purified by paper chromatography or TLC using the same solvent system. The compound was stored at -20°C in the dark.

Fig. 1

Fig. 1. Effect of iron concentration on the growth of *A. brasilense*. *A. brasilense* was grown overnight in FMM without iron, washed with the same medium and reinoculated to an OD_{590} of 0.1 into the same medium containing no added iron (○) or $10\ \mu\text{M-FeCl}_3$ (△). The cells were grown in a shaker-incubator at 32°C for 30 h. Inset: concentration of spirilobactin in the cell-free supernates after 30 h growth in FMM containing different concentrations of added FeCl_3 . The concentration of spirilobactin was measured as described in Methods.

Fig. 2

Fig. 2. Reversal of EDDA-induced growth inhibition by spirilobactin. *A. brasilense* was grown in LBS medium. EDDA ($50\ \mu\text{g ml}^{-1}$) was added after 3 h growth and spirilobactin was added after a further 4 h. ○, Control (no additions); □, EDDA only added; △, EDDA and spirilobactin added.

Partial characterization of spirilobactin

The UV spectra of spirilobactin in water and in $0.1\ \text{M-HCl}$ were identical with peaks at 308, 243 and 206 nm. Spirilobactin was completely hydrolysed in $6\ \text{M-HCl}$ (110°C , 24 h) under an atmosphere of nitrogen. The hydrolysate was diluted with water and extracted with ethyl acetate. The ethyl-acetate-soluble fraction yielded a ferric-chloride-positive, ninhydrin-negative material that fluoresced under UV illumination. It was identified as DHBA by comparison with the authentic compound by spectrophotometric, fluorometric and chromatographic techniques (solvent systems A and D). The aqueous fraction when chromatographed with solvent systems A, B and C showed no fluorescence but gave two ninhydrin-positive spots. These three components could also be separated by Dowex 50 chromatography eluting with increasing concentrations of HCl. The ninhydrin-positive materials were identified by paper chromatography as serine and ornithine and their identity was further confirmed by amino acid analysis (Beckman Multichrom) and by mass spectrum analysis. As $1\ \mu\text{mol}$ of spirilobactin (based on the molar concentration of DHBA residues as determined by the Arnow test) yielded approximately $1\ \mu\text{mol}$ each of serine and ornithine on complete acid hydrolysis, it was concluded that the three constituents, i.e. DHBA, serine and ornithine, were present in equimolar amounts.

Spirilobactin-mediated iron uptake

Transport of free iron was eliminated by the presence of $0.2\ \text{mM-NTA}$ in the uptake buffer. Uptake of iron from the $^{59}\text{Fe(III)}$ -spirilobactin complex was shown only by cells starved of iron, indicating the induction of a spirilobactin-dependent iron transport system in *A. brasilense*. The uptake of ^{59}Fe by the induced cells was linear with time for about 25–30 min when a saturating concentration of $^{59}\text{Fe(III)}$ -spirilobactin was used (Fig. 3). Transport was mediated by an active process and no uptake occurred at $0-4^\circ\text{C}$.

Fig. 3

Fig. 3. Uptake of ^{59}Fe from the $^{59}\text{Fe}(\text{III})$ -spirilobactin complex by *A. brasilense* RG. ^{59}Fe uptake by induced cells at 32 °C (○) and 4 °C (□), and by uninduced cells at 32 °C (●) was measured as described in Methods. Δ, Induced cells preincubated at 32 °C in succinate-free uptake buffer for 45 min and ^{59}Fe uptake measured in the same succinate-free buffer at 32 °C.

Fig. 4

Fig. 4. Kinetics of induction of iron-regulated outer membrane proteins of *A. brasilense* RG. The iron-regulated proteins were induced by the addition of EDDA ($50 \mu\text{g ml}^{-1}$) to exponentially growing cells of *A. brasilense* RG in LBS medium; the outer membrane proteins were isolated from cells harvested at different times after EDDA addition. SDS-PAGE (8%, w/v, acrylamide) of the outer membrane proteins was done as described in Methods. A, Control cells; B, C, D, E, EDDA added and cells collected after 1, 2, 3 and 5 h, respectively.

In the accompanying paper (Bachhawat & Ghosh, 1987) we demonstrated the appearance of four iron-regulated proteins in the outer membrane of *A. brasilense* RG grown in iron-deficient media. It is conceivable that one of these four proteins is the receptor (carrier) for the $^{59}\text{Fe}(\text{III})$ -spirilobactin complex. To determine the kinetics of induction of these proteins, the iron chelator EDDA was added to exponentially growing cells of *A. brasilense* RG and outer membranes were isolated from cells harvested at intervals. Within 3 h of addition of EDDA, which is about one and a half generation times, the proteins were fully induced (Fig. 4).

Kinetics of transport

The optimum pH value for iron transport was determined by measuring uptake in uptake buffers adjusted to pH values between 6.2 and 8.5. The optimum pH value for transport was 7.0. Uptake was also measured under standard conditions but at incubation temperature from 25 to 46 °C. Transport showed a broad temperature optimum with a peak at 35 °C. To determine the binding constant of the transport system, iron uptake rates were measured with increasing concentrations of the $^{59}\text{Fe}(\text{III})$ -spirilobactin complex. The initial uptake rate was dependent on the concentration of the complex; the rate approached a maximum value when the concentrations were raised indicating that this was a carrier- or receptor-mediated transport process. From Lineweaver-Burk plots (not shown) a K_m value of $0.23 \mu\text{M}$ and a $V_{\max}$ of $0.27 \text{ nmol min}^{-1} (\text{mg cell protein})^{-1}$ were determined.

Energy requirements

Depletion of endogenous substrate by shaking the induced cells without succinate in phosphate buffer for 45 min before determining transport greatly reduced the uptake rate, particularly after the first 10 min (Fig. 3).

Since *A. brasilense* failed to show uptake of ^{59}Fe from the $^{59}\text{Fe(III)}$ -spirilobactin complex when depleted of endogenous substrate, it was of interest to know the energy source that drives the transport process. To determine this, the effect of several inhibitors was investigated. The protonophore CCCP was an extremely potent inhibitor of transport; KCN and the protonophore, 2,4-DNP also caused a significant reduction of transport. These results suggested that an energized membrane was required for driving transport. ATP was not directly required for transport, since 4 mM-arsenate caused no reduction in transport (at lower concentrations a slight stimulation of the activity was observed). Almost complete inhibition of transport by 0.1 mM-*p*CMB suggested that sulphhydryl groups played an essential role at some point during the uptake process.

Uptake of $^{59}\text{Fe(III)}$ -spirilobactin by *A. lipoferum* and *A. amazonense*

To determine whether other members of the genus *Azospirillum* could use the siderophore produced by *A. brasilense*, the uptake of ^{59}Fe by the three different species of *Azospirillum* was compared. Iron-deficient cells of *A. lipoferum* cells could transport ^{59}Fe from the $^{59}\text{Fe(III)}$ -spirilobactin complex at a rate 3-fold lower than *A. brasilense* but no uptake of ^{59}Fe was observed in the case of *A. amazonense*.

DISCUSSION

The results described in this paper demonstrate that *A. brasilense* releases a catechol-type siderophore into the growth medium when subjected to iron deficiency. This siderophore, which we call spirilobactin, could reach a concentration as high as 200 nmol (based on its DHBA content) (ml culture supernate) $^{-1}$ when the bacteria were starved of iron. The siderophore was purified by simple chromatographic techniques but, as the compound is a derivative of catechol, extreme care was necessary during purification to prevent its spontaneous oxidation and polymerization in the presence of light and atmospheric O_2 . Although it could be demonstrated that spirilobactin contains only three identifiable components (DHBA, serine and ornithine) in a 1:1:1 molar ratio, elucidation of its complete structure will entail further studies.

The isolation and purification of spirilobactin allowed us to demonstrate a high-affinity, iron uptake system in *A. brasilense* where the siderophore serves as an extracellular carrier of iron. Induced synthesis of the receptor protein (in the outer membrane) for the iron(III)-spirilobactin complex and the extracellular siderophore both seemed to occur simultaneously when *A. brasilense* cells were grown in iron-deficient medium. This high-affinity iron uptake system may be extremely useful for scavenging iron from plant tissue when *A. brasilense* infects plant roots and consequently becomes dependent on iron from the host for survival.

Spirilobactin-dependent iron uptake by *A. brasilense* was an active process that required an energized membrane. The strong inhibition by CCCP and 2,4-DNP suggested that the proton gradient was driving transport. ATP hydrolysis did not seem to be directly coupled to transport. In *E. coli*, ferric-enterobactin transport ($K_m = 0.1 \mu\text{M}$) depends on the proton gradient component of the protonmotive force (Pugsley & Reeves, 1977) while iron uptake through the ferrichrome system ($K_m = 0.15\text{--}0.25 \mu\text{M}$) depends primarily on the electrical component (Negrin & Neilands, 1978). Our results indicate that ferric-spirilobactin transport has characteristics similar to the ferric-enterobactin transport of *E. coli*.

The ability of *A. lipoferum* to take up iron from iron(III)-spirilobactin, although much less efficient, suggests the existence of a spirilobactin-type, high-affinity iron transport system in this species of *Azospirillum*; however, we were unable to detect the spirilobactin-dependent iron-transport system in *A. amazonense*. The significance of these observations is not clear at this stage.

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