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Indirect utilization of the phytosiderophore mugineic acid as an iron source to rhizosphere fluorescent *Pseudomonas*

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The phytosiderophore mugineic acid (MA) was studied as a source of iron for rhizosphere fluorescent pseudomonads. ^{55}Fe supplied as Fe–MA was taken up by *Pseudomonas putida* WCS358, B10 and St3 grown under iron deficient conditions. The uptake decreased when the bacteria were grown in the presence of iron. However, no differences in uptake were observed when a siderophore deficient mutant was tested. Since ligand exchange between pseudobactin and MA was shown to occur rapidly with a half-life of 2 h, MA mediated iron uptake probably proceeds through this indirect mechanism. The ecological implications of these findings are discussed.

Keywords: iron uptake, mugineic acid, phytosiderophore, *Pseudomonas putida*

Introduction

Iron is an essential element for most organisms. Due to the low solubility of its oxides, plants and microorganisms have developed various strategies operating to obtain sufficient supply of this essential element. Under iron deficiency many microorganisms excrete siderophores which strongly and specifically bind Fe^{3+} (Neilands 1982). Dicoteledonous plants excrete protons, reductants and activate a reductase which reduces chelated Fe^{3+} to Fe^{2+} which is then absorbed by the root (Romheld 1987). Gramineous plants produce phytosiderophores, which are iron solubilizing biosynthetic chelates derived from nicotianamine (Nomoto *et al.* 1987, Shojima *et al.* 1990). These compounds are known as mugineic acids (MAs). Some divergences exist as to the stability constant (log K) of MA with Fe^{3+}. One study has determined it to be 18.1 (Nomoto *et al.* 1987), while in another a log K of 32 was reported (Murakami *et al.* 1989). MAs were shown to supply iron to iron stressed monocot plants, increasing the chlorophyll content of the leaf (Mino *et al.* 1983). MA excretion and uptake take place at the root tip (Mihashi & Mori 1989) which is not as heavily colonized by microorganisms as the elongation zone (van Vuurde & Schippers 1980). This can somewhat protect MAs from microbial breakdown which has been shown to occur rapidly (Watanabe & Wada 1989). Bacteria which can thrive on MAs as their sole carbon source have been isolated and a characterized strain belongs to the genus *Pseudomonas* (Watanabe & Wada 1989). Pseudomonads are versatile bacteria as to their use of organic compounds as a carbon source, many of them having the ability to decompose a great number of substrates (Stanier *et al.* 1966).

Fluorescent pseudomonads are common rhizobacteria. They produce pseudobactin (PSB)-type siderophores which are not readily available as an iron source to other rhizosphere bacteria (Jurkevitch *et al.* 1992), but they are able to utilize a wide variety of other siderophores. Thereby, fluorescent pseudomonads gain an ecological advantage (Buyer & Sikora 1990, Jurkevitch *et al.* 1992). Since monocots may release phytosiderophores to the rhizosphere when stress prevails, it is of interest to study the availability of such chelators as iron carriers to pseudomonads. Elaboration of the interactions of

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Fe^{3+}–MA complexes with fluorescent pseudomonads was the aim of this study.

Materials and methods

Bacterial strains

Pseudomonas putida WCS358, its siderophore minus (*sid*$^-$) Tn*5* induced mutant JM218 and *P. putida* B10 were obtained from of P. Weisbeek (Utrecht, The Netherlands) and J. Buyer (Beltsville, MD), respectively. *P. putida* St3 has been isolated in our laboratory.

Siderophores

MA and PSB were purified as previously described (Mori *et al.* 1987, Jurkevitch *et al.* 1992).

Plate tests

The antibiosis plate test was carried out as described by Buyer & Leong (1986) with 0.6 ml of soft agar inoculated with approximately 10^3 c.f.u. poured over rhizosphere simulating medium (RSM) (Buyer *et al.* 1989) in 5 cm diameter Petri dishes. Then, 20 μl of a 10^{-3} M solution of the tested desferri compound was dropped onto a 6 mm diameter Whatman paper disk. The plates were incubated at 30 °C and checked after 24 h.

For the iron–siderophore complex utilization test, the bacteria were grown in liquid RSM and 10^3 c.f.u. ml^{-1} were plated on RSM plates containing 201 μM EDDHA (Buyer *et al.* 1989) treated as described by Rogers (1973). Then, 10 μl of a 10^{-4} M solution of the iron–siderophore complexes was dropped on a Whatman paper disk. The plates were incubated as above.

Growth curves

The *sid*$^-$ strain JM218 was used to test the efficiency of bacterial growth promotion in an iron-free liquid medium. HEPES–succinate medium (HSM), (succinate, 4 g l^{-1}; K_2HPO_4, 0.2 g l^{-1}; $(NH_4)_2SO_4$, 1 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 g l^{-1}; HEPES, 11.91 g l^{-1}) was batch treated for 4 h with Chelite N resin (10 g l^{-1}; Serva, Germany) decanted and filtered through a 0.45 mm filter. The pH was then raised to 7.2 by adding 10N NaOH and the medium was autoclaved. Double-distilled water (DDW) was used and the glassware was washed with 6N HCl followed by thorough rinsing with DDW.

Polypropylene test tubes (50 ml) were filled with 10 ml of the Chelite-treated HSM. The Fe^{3+} siderophores were added to a final concentration of 10^{-6} M and shaken in an inclined position at 28 °C. Samples were taken at desired intervals and turbidity at 620 nm was recorded. Each treatment was performed in triplicate.

^{55}Fe uptake

The procedure described by de Weger *et al.* (1988) was used with minor modifications. The bacteria were grown in liquid RSM to an A_{620} of 0.6–0.7, centrifuged for 15 min at 2500 r.p.m., then resuspended in liquid RSM to a final A_{620} of 0.5 and incubated for 30 min in a water bath at 28 °C. In some experiments, $FeCl_3$ (10^{-5} M) was added to achieve iron repleted conditions. The labelled Fe^{3+}–MA complex was added to a final concentration of 1 μM. Aliquots (0.5 ml) were taken in duplicate, layered onto a mixture of octoil:dibutyl phtalate (1:2) and centrifuged as described. Cell growth during the time of the assay was measured by absorbance and accounted for in dry weight measurements. Counting was performed on a Beckman LS 1801 counter (USA).

Ligand exchange

Fe–MA (50% saturated, 2×10^{-5} M MA, 10^{-5} M Fe) was dissolved in BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] buffer, 0.02 M, pH 7 and was challenged by an equimolar concentration of free PSB St3 (10^{-5} M). Temperature was kept constant at 30 °C and the formation of the Fe–PSB complex was followed by measuring the absorbance of the Fe–PSB complex at 450 nm (Meyer & Abdallah 1978) on an HP8452A spectrophotometer (Hewlett-Packard, USA). At this wavelength Fe–MA virtually shows no absorbance (Nomot *et al.* 1987). Absorption was measured every 10 s for 660 s and then every 240 s. In the reverse reaction, Fe–PSB (10^{-5} M) was challenged with an equimolar concentation of free MA under the same conditions.

Results

Growth promotion tests

Three strains of *P. putida* were grown in iron-deficient RSM, which simulates the composition of the rhizosphere (Buyer *et al.* 1989). The bacteria were spread on RSM plates containing EDDHA and growth promotion tests with Fe–MA as the sole iron source were performed. As shown in Table 1, Fe^{3+}–MA promoted the growth of all three strains, although B10 lagged behind St3 and WCS358. After 18 h growth was apparent in strains St3 and WCS358 but significant growth of B10 developed only after

Table 1. Growth promoting effect of Fe^{3+}–MA (10^{-4} M, 10 mM) on *P. putida* strains St3, WCS358 and B10 on RSM + EDDHA plates

	Strains[a]		
Time (h)	St3	WCS358	B10
18	17	14	11[b]
40	40	37	23

[a]Diameter of growth around a Whatman paper disk of 6 mm diameter at 28 °C.
[b]Very small colonies.

40 h. In antiobiosis-like plate tests in which the desferri MA was used none of the three strains was inhibited by MA.

The growth-promoting effects of Fe–MA were also studied in a Chelite-treated HSM liquid medium with the siderophore deficient mutant JM218. The Fe–MA complex was utilized as a source of iron, strongly promoting the growth of the bacterium (Figure 1). These results indicate that the Fe^{3+}–MA complex is used to provide the bacterium with iron.

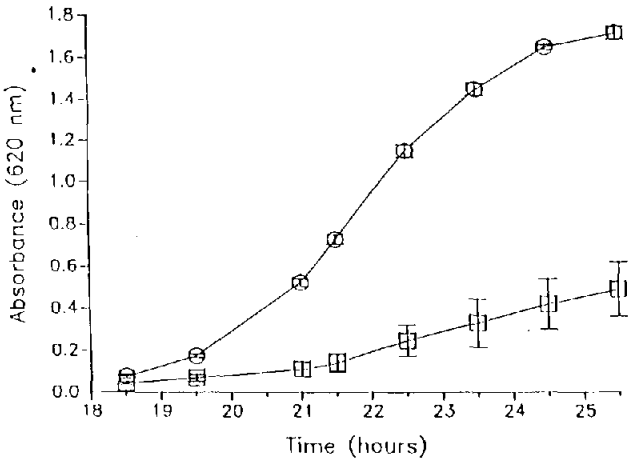

Figure 1. Growth of mutant JM218 in liquid Chelite-treated HSM with Fe–MA as the sole source of iron (○). Control (□), no added iron. Bars represent standard errors.

^{55}Fe uptake

In order to confirm utilization of Fe–MA, ^{55}Fe uptake from ^{55}Fe–MA was demonstrated. After 20 min an uptake of 0.92, 1.34 and 1.7 nmol Fe mg^{-1} dry weight was measured in strains St3, B10 and WCS358, respectively (Figure 2). Uptake rates of 0.065, 0.096 and 1 nmol Fe $min\,mg^{-1}$ dry weight were calculated from the linear part of the uptake curve. Uptake was completely abolished in the presence of cyanide anions indicating an active process (Figure 2). The influence of the iron status of the cells was investigated by culturing the bacteria in an iron replete medium. Under these conditions no PSB is produced. Uptake of ^{55}Fe mediated by MA in the iron-replete cells was decreased by about 60, 70 and 70% in St3, B10 and WCS358, respectively (Figure 2). This decrease is not as important as usually seen in the case of PSB-mediated iron transport (de Weger *et al.* 1988, Hohnadel & Meyer 1988). The intensity of uptake in iron deficient cells as well as the uptake rates obtained are similar to data published for PSB-mediated iron uptake (de Weger *et al.* 1988, Hohnadel & Meyer 1988). In order to clarify the reason for the high residual uptake in iron-replete cells, the Tn5 induced *sid*$^-$ mutant JM218 was challenged with $^{55}Fe^{3+}$–MA after cultivation in an iron deficient or in an iron sufficient medium. As shown in Figure 2, MA mediated ^{55}Fe uptake in strain JM218 was independent of the iron status of the cell. Moreover, the uptake curve is similar to the one obtained with the

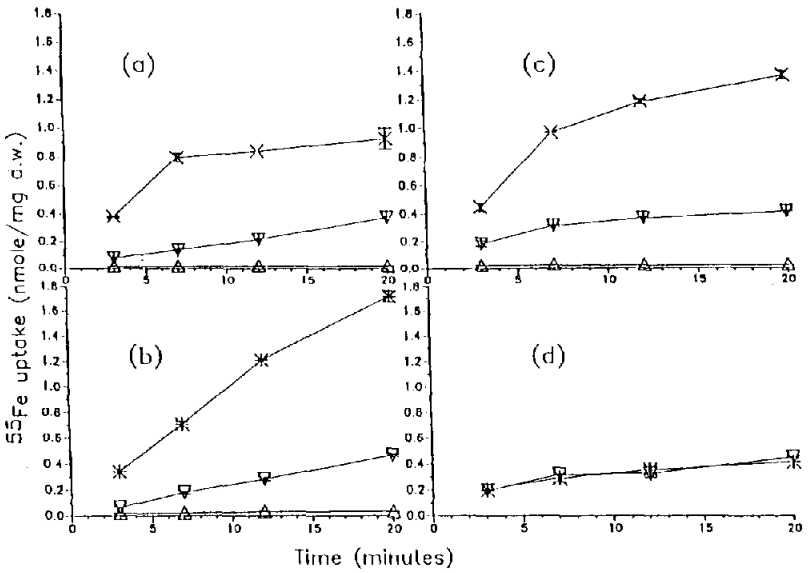

Figure 2. ^{55}Fe uptake mediated by MA in *P. putida* strains St3 (**a**), WCS358 (**b**), B10 (**c**) and the siderophore deficient mutant JM218 (**d**). (∗) Iron deficient cultures, (▽) iron sufficient cultures, (△) plus 10 mM CN^-, added 20 min prior to the beginning of the experiment.

iron-replete cells of the wild-type parent WCS358 (Figure 2).

Ligand exchange

Strains St3, B10 and WCS358 excrete PSB when grown under iron deficiency. During the time of the experiment (50 min from resuspension of the cells in fresh RSM to the end of the uptake test) excretion of PSB occurs. This was proved by centrifugating the cells and testing the supernatant which showed a spectrum typical of free PSB (Meyer & Abdallah 1978). From this spectrum, a final concentration of about 2×10^{-5} M was estimated. Therefore, the possibility of ligand exchange between MA and PSB excreted by the wild-type bacteria exists. When the Fe^{3+}–MA complex was challenged by free PSB, almost total ligand exchange was achieved after 10.25 h (Figure 3). The spectrum of the resulting Fe–PSB was similar to that of fully chelated PSB. A half-life of 2 h was calculated using the regression line obtained from this data. Under these conditions about 3.4×10^{-6} M of FE–PSB could be formed by iron exchange between MA and the St3 PSB in 1 h. Fully chelated PSB was challenged with an equimolar concentration of MA. In that case, virtually no ligand exchange could be detected (Figure 3).

Discussion

In this study, it is shown that utilization of the Fe^{3+}–MA complex as a source of iron by fluorescent pseudomonads occurs via an indirect mechanism. Since uptake of ^{55}Fe from Fe–MA in mutant JM218 was not enhanced by iron depletion and since the kinetic data show that the high uptake rates observed in the wild-type strains can be the result of ligand exchange, we assume that the Fe^{3+}–MA complex is not recognized as a siderophore. Rather, it is perhaps metabolized for other purposes, possibly as a carbon source. *P. putida* can grow on any of 18 amino acids, except methionine, as a sole carbon source (Jacoby 1964). This amino acid has been shown to be used as a sole nitrogen source by *P. aeruginosa* (Kay & Gronhund 1969a). *P. aeruginosa* possesses constitutive permeases for the active transport of amino acids, showing uptake rates and K_ms of 0.2 to 8 nmol min mg^{-1} dry weight and 10^{-7} to 10^{-6} M, respectively (Kay & Gronhund 1969b).

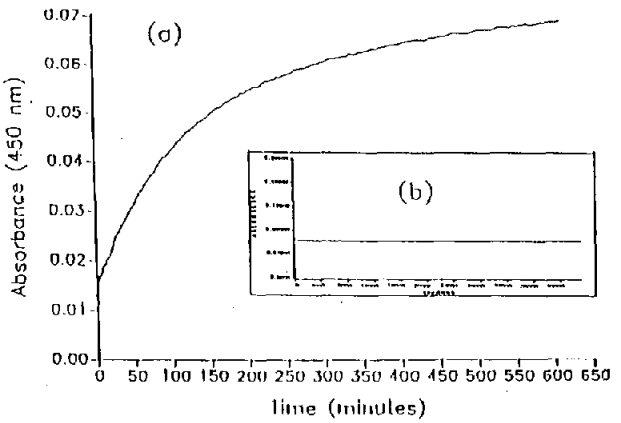

Figure 3. Ligand exchange between Fe^{3+}–MA and PSB from strain St3 (**a**) and between Fe^{3+}–PSB and MA (**b**) in BES buffer (0.02 M, pH 7) at 30 °C.

The ecological significance of these findings deserves attention. As previously reported (van Vuurde & Schippers 1980, Romheld 1991), the spatial repartition of the MA release and of the high bacterial activity may protect phytosiderophores from being quickly degraded by the bacterial flora. Using plants inoculated as to achieve different population levels or different bacterial compositions, Bar-Ness *et al.* (1992a) were able to demonstrate that bacterial siderophore-mediated iron uptake in roots is enhanced in inoculated as compared with sterilized roots. By using confocal microscopy, this activity was shown to take place 40 mm from the root tips in maize, in a zone where dense mucigel was observed (Bar-Ness *et al.* 1992b). Similarily, it was recently shown that microbial inoculation of maize roots decreases hydroxymugineic acid-mediated iron uptake and translocation (Crowley *et al.* 1992). Physical separation between the zones of active release and uptake of MAs and the heavily populated root segments could protect MA not only from microbial degradation, but also from competition for iron with PSB and, probably, other bacterial siderophores. This spatial difference coupled with the very efficient uptake system of Fe^{3+}–MAs (Romheld 1991) certainly could provide the basis for their high activity as iron carriers in gramineous plants.

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