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Microbial adaptation to iron: a possible role of phosphatidylethanolamine in iron mineral deposition

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Pseudomonas fluorescens **multiplied in a minimal mineral medium supplemented with iron(III) (5 mM) complexed to citrate, the sole source of carbon, with no apparent diminution in cellular mass. Atomic absorption studies of different cellular fractions and supernatant at various growth intervals revealed that the trivalent metal was initially internalized. At approximately 41 h of incubation, the soluble cellular extract contained 9.5% of the iron originally found in the growth medium. However, as bacterial multiplication progressed, most of the metal was deposited as an extracellular insoluble gelatinous residue. Phosphatidylethanolamine appeared to be an important organic constituent of this precipitate. X-ray fluorescence and diffraction studies revealed that iron(III) was deposited as amorphous hydrated oxide. Scanning electron microscopy and energy dispersive X-ray microanalysis of the pellet aided in the identification of irregular shaped bodies rich in iron and oxygen that were associated with carbon-containing elongated structures. Examination of the bacterial cells by a transmission electron microscope equipped with an electron energy loss spectrometer indicated the deposition of iron within the cells.**

Keywords: homeostasis, iron, mineral, phosphatidylethanolamine

Introduction

Iron is an essential element for the normal functioning of most living systems owing to its participation in a wide variety of biological activities (Crichton 1990). This importance is primarily due to its ability to undergo oxidation reduction reactions. However, the redox chemistry of iron lends itself to the formation of harmful free radicals. Thus in order to circumvent the adverse effects of this essential metal, most organisms control the level of free iron (Halliwell & Gutteridge 1984). In humans, transferrin and ferritin are pivotal in the regulation of iron (Theil 1987). Perturbation of iron homeostasis is known to trigger various pathological complications. While increased absorption of iron during idiopathic hemachromatosis leads to severe tissue injury, a deficiency in the trivalent metal results in anemia (Grootveld *et al.* 1989). No mechanism of iron excretion in humans is known. In the microbial world bacterioferritin and siderophores appear to play a central role in the attainment of iron balance. The former is involved in storage while the latter helps in the acquisition of the trivalent metal during iron starved situations (Briat 1992, Williams 1990, Appanna *et al.* 1984).

Elevated bioavailability of metals induced by industrial activities and acid rain has become a major cause of concern due to the negative impact of metal pollutants on all life forms. Iron(III) that is released from insoluble precipitate may be mobilized by citric acid, a naturally occurring tricarboxylic acid that is also found in industrial and domestic wastes (Francis *et al.* 1992). This tricarboxylate moiety is known to have a high affinity for the trivalent metal (Martin 1986). Thus it is important to evaluate the mineralization and the impact of the iron(III)–citrate complex on living systems. Although citrate has been implicated in iron transport in numerous microbes, citrate–metal complexes are not known to be readily utilized as carbon source (Lauff *et al.* 1990). Furthermore the biodegradability of these complexes appears to be influenced by the nature of the binding between the ligand and the metal (Francis *et al.* 1992). Thus the resistance and/or the facility of the citrate–metal complexes to biodegradation may be an important factor in metal biogeochemical cycles and metal waste management.

In this study we have investigated the biodegradability

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of iron(III) bound citrate by the bacterium *Pseudomonas fluorescens*. The tricarboxylic acid was the sole source of carbon in the minimal growth medium. Here the ability of the microbe to metabolize iron(III) citrate and to regulate its intracellular iron content are reported. The attainment of iron homeostasis by the deposition of hydrated iron(III) oxide is also discussed. It appears that the metal is initially internalized and then secreted as oxide(s) associated with phosphatidylethanolamine.

Materials and methods

Media and bacterial cultivation

P. fluorescens ATCC 13525 was obtained from the American Type Culture Collection and was maintained at 4 °C by monthly subculture on a mineral citrate medium solidified by the inclusion of 2% agar. The liquid culture contained Na_2HPO_4 (2.4 mg), KH_2PO_4 (1.2 mg), NH_4Cl (0.09 g), $MgSO_4 \cdot 7H_2O$ (0.2 g) and citric acid (4.0 g) per litre of deionized double-distilled H_2O. Trace elements were added in the following concentrations: $Zn(NO_3)_2 \cdot 6H_2O$ (0.07 mg l^{-1}), $CaCl_2$ (0.11 mg l^{-1}), $CuCl_2 \cdot 2H_2O$ (0.017 mg l^{-1}), and $Na_2MoO_4 \cdot 2H_2O$ (0.024 mg l^{-1}) and $FeCl_3 \cdot 6H_2O$ (0.54 mg l^{-1}). Iron(III) (5 mM) was supplemented as ferric citrate and/or chloride salts. The medium without added iron(III) served as control. The pH of the media was adjusted to 6.8 with dilute NaOH.

The media were dispensed in 200 ml amounts in 500 ml Erlenmeyer flasks and inoculations were made with 1 ml of stationary phase cells grown in a control mineral citrate medium (Al-Aoukaty *et al.* 1991). The cultures were aerated on a gyratory waterbath shaker model G76 (New Brunswick Scientific) at 26 °C at 140 r.p.m. At timed intervals, cells were harvested by centrifugation at 10 000 *g* for 20 min at 4 °C. Following disruption of the cells with 0.5 M NaOH in boiling water, microbiol growth was monitored by measuring soluble protein by the method of Lowry *et al.* (1951) and Bradford (1976). Bovine serum albumin was used as the standard. The spent fluid devoid of bacterial cells was analyzed for its protein and carbohydrate content, respectively (Bradford 1976, Dubois *et al.* 1956). The pH was also recorded with the aid of a Fisher pH meter model 610A. Citrate utilization was assayed with citrate assay kit (Moellering & Gruber 1966).

Iron measurement

At various periods of growth, iron metabolism in *P. fluorescens* was assayed on a Perkin-Elmer atomic absorption spectrometer model 703. The bacterial cells, following centrifugation of the culture broth at 10 000 *g* were sonicated to afford the soluble and particulate fractions. The exocellular precipitate that appeared at later stages of growth was separated either by centrifugation at 1000 *g* and/or by allowing the culture flasks to stand undisturbed for 15–30 min. The iron in these fractions was digested with concentrated HNO_3 and analyzed by atomic absorption spectrometer. $FeCl_3$ was used as the standard.

Pellet isolation and identification

After an incubation period of 62 h, culture flasks were left to stand undisturbed for 15–30 min to allow for the separation of cells and exocellular precipitate. The cloudy layer containing the bacterial cells was siphoned off while the lower reddish-brown fraction was centrifuged at 1000 *g*. The pellet was resuspended in 150 ml of distilled water and centrifuged at 1000 *g*. This procedure was repeated twice to ensure bacteria-free pellets. The precipitate was hydrolyzed and examined for its iron, protein and carbohydrate. Lipids from the pellet were extracted with a mixture of CH_3OH:$CHCl_3$:H_2O (2:1:0.8). The lipids were then placed as spots on thin layer silica gel plates (Whatman, Germany) and resolved by ascending chromatography using chloroform:methanol:ammonium hydroxide (65:25:5 v/v). The lipids were visualized with I_2 vapor, ninhydrin and molybdate reagents (Kates 1988). Following the removal of the lipids the pellet was suspended in water and dialyzed extensively (MWCO 12–14 kDa). The reddish-brown powder obtained following lyophilization was subjected to various analytical studies. The nature of the iron in the mineral was determined by the ferrozine assay in the presence and absence of hydroxylamine hydrochloride in both the pure and freshly isolated pellets (Stookey 1970). Fourier transformed infrared (FTIR) spectra were recorded on a Bomen Michelson-100 FTIR. X-ray fluorescence analysis was performed on a Philips PW1404 automatic, sequential spectrometer. A methane/argon gas mixture was used in the flow proportional counter and a rhodium X-ray tube was employed throughout this investigation. The analyzing crystals were LIF 200 and PX1. Intensities are given in kilocounts per second (kc.p.s.) and peak shifts in two theta degrees ($2\theta°$). X-ray diffraction analysis data were obtained with a 114.6 mm Straumanis-type Camera on a Philips PW1010 generator. A JEOL model 6400 scanning electron microscope (SEM) fitted with a TN5500 energy dispersive X-ray microanalytical system (INCO, Sudbury, Ontario, Canada), was utilized to image the morphological features and to record the elemental composition of this biomineral.

Transmission electron microscopic studies

Microbes harvested at various incubation periods were washed twice with 0.85% NaCl solution and were fixed in 3% glutaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). Post-fixation was achieved in 1% osmium tetroxide dissolved in 0.1 M phosphate buffer for 1.5 h. After washing twice with double-distilled water the samples were further fixed and stained in 2% uranyl acetate and embedded in 2% agar. The solidified agar was sliced into small blocks, dehydrated in a series of alcohol, embedded in Epon 812 resin and cured at 60 °C for 36 h. Thin sections were examined with or without further lead citrate staining by electron energy loss spectroscopy (EELS) in a Zeiss 902A transmission electron microscope (TEM).

Results

When subjected to 5 mM of iron(III), complexed to citrate as the sole source of carbon, *P. fluorescens* did not experience any inhibition in the rate of cellular growth. There was no significant variation in the biomass at the stationary phase of growth in control and metal supplemented cultures. When cells isolated at the stationary phase of growth from iron-rich culture were inoculated in a fresh medium with 5 mM iron, an increment of growth rate was observed (data not shown). Citrate was rapidly consumed and at about 40 h of incubation none of this tricarboxylic acid was detected in the spent fluids (Figure 1). No significant change in protein and carbohydrate contents of the spent fluid was observed in control and metal stressed cultures. At the stationary phase of growth the level of carbohydrate was 150 $\mu g\,ml^{-1}$ of culture (glucose equivalent) in the iron-rich medium while that in the control was 80 $\mu g\,ml^{-1}$ of culture. The protein concentration in the spent fluids amounted to approximately 25 $\mu g\,ml^{-1}$ in control and metal supplemented media. The pH of the supernatant at the stationary phase of growth increased to 8.2–8.9 in both media.

At different growth intervals the metabolic fate of iron was monitored by atomic absorption spectrophotometry. As growth progressed, the level of soluble iron in the supernatant fluid showed a slight decrease. Up to 30 h of incubation, only a very small amount of iron was detected in the soluble extract and only some of the trivalent metal appeared to be associated with the cell debris. However, at 41 h of growth, the former component comprised 9.5% of the total iron in the medium. Further incubation led to the deposition of a reddish-brown gelatinous mass, where most of the initial iron in the growth medium was sequestered (Figure 2). This deposit was absent if the inoculated medium was sterilized prior to incubation and if the pH of the uninoculated medium was raised to 10.0.

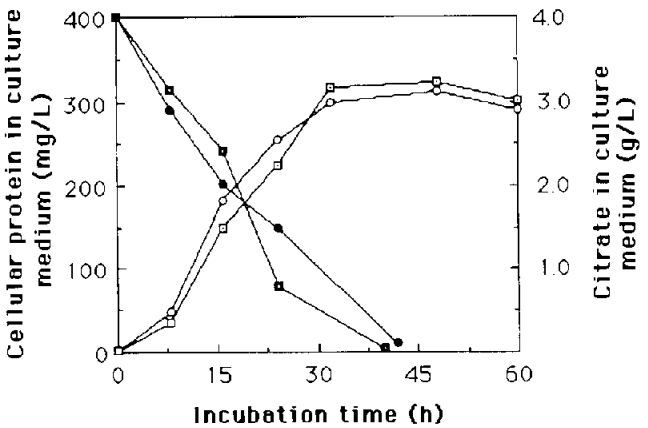


Figure 1. Bacterial growth and citrate utilization at various growth periods. Mean values of three experiments are reported. Standard deviation ranged from 0.01 to 0.09 and 2.01 to 6.08 for citrate and protein analyses, respectively. (○) Cellular protein from control cultures, (□) cellular protein from iron supplemented cultures, (●) citrate utilization in control medium and (■) citrate utilization in iron supplemented cultures.

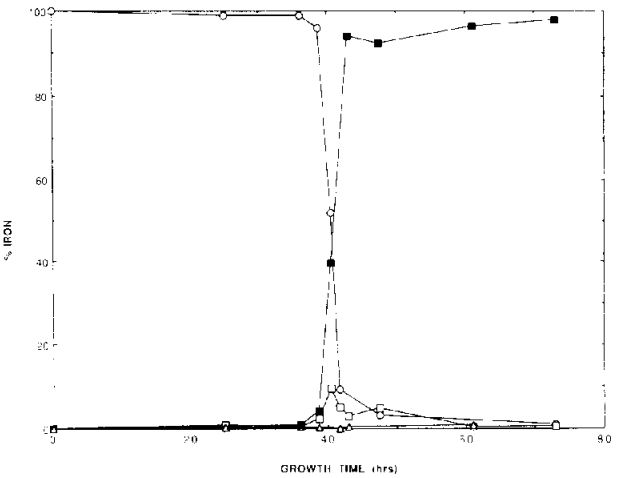


Figure 2. Iron metabolism in *P. fluorescens* ATCC 13525. Mean values of five experiments are reported. Standard deviation ranged from 1.22 to 2.18. (○) Supernatant, (□) cytoplasm, (■) pellet and (△) cell debris.

The reddish-brown gelatinous residue was isolated by centrifugation. It was hydrolyzed and analyzed for its protein and carbohydrate contents. No detectable amount of these moieties was discerned. The chloroform:methanol extract of the pellet was subjected to thin layer chromatographic analyses. The polar solvent system helped resolve a major band with a R_F value of 0.42 that responded positively to ninhydrin and ammonium molybdate sprays. This spot was found to co-migrate with standard phosphatidylethanolamine. The dark brown powder that was obtained from the gelatinous pellet following the extraction of lipids was freeze dried and examined by X-ray fluorescence spectroscopy. Two peaks at 51.75 $2\theta°$ and 57.5 $2\theta°$ attributable to the K_β and K_α fluorescence of iron were detected (Figure 3). Only trace amounts of phosphorus (14.9 $2\theta°$) were evident (White & Johnson 1970). The FTIR spectrum revealed a band at 3377.94 cm^{-1}

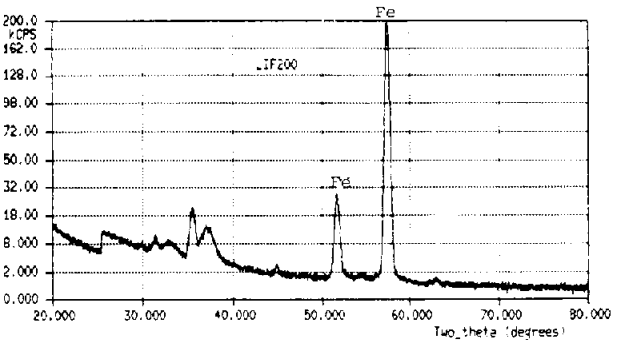
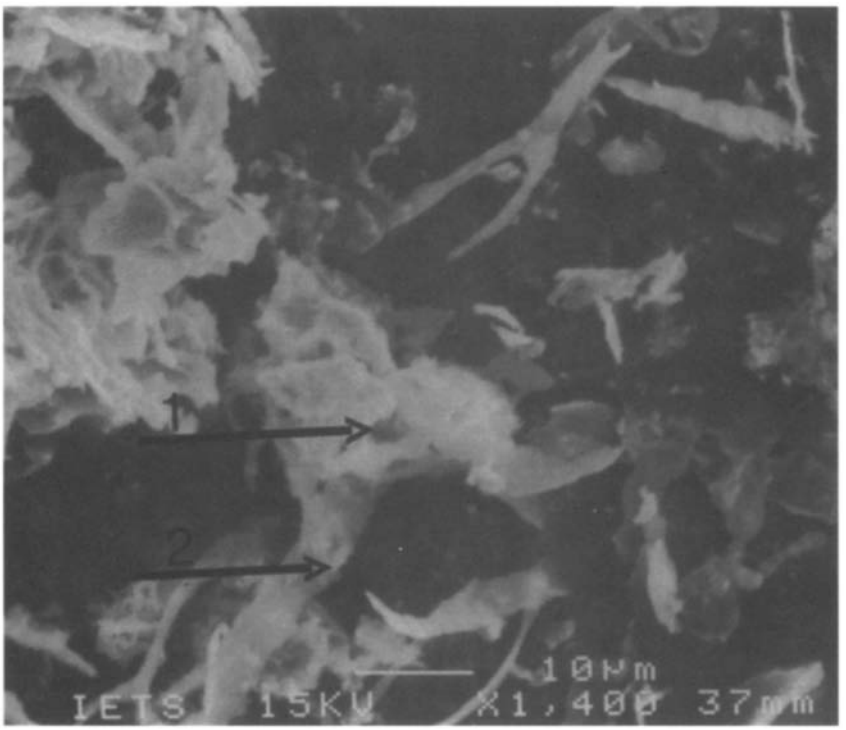


Figure 3. X-ray fluorescence spectrum of the iron mineral produced by *P. fluorescens* grown in an iron(III) supplemented medium.

indicative of an O–H stretching vibration (data not shown) X-ray diffraction analysis of both the mineral with and without lipid moieties revealed no defined patterns, thus confirming the amorphous nature of the iron mineral. Examination of the precipitate with ferrozine in the presence of hydroxylamine aided in the identification of the metal primarily as iron(III). Imaging of the gelatinous mass with a SEM equipped for energy dispersive X-ray microanalysis revealed iron- and oxygen-rich irregular shaped bodies associated with elongated thread-like, carbon-containing morphological features (Figure 4). The presence of iron in the bacterial cells was monitored by a TEM equipped for EELS. The cells harvested at 37 h of incubation were found to contain electron dense bodies that were attributable to iron. The metal appeared to be sequestered within the bacterial cells. A bacterial cell with iron deposits was also visualized in its dividing state (Figure 5).

Discussion

The data in this report demonstrate the ability of *P. fluorescens* to achieve iron(III) homeostasis via the elaboration of an exocellular iron-containing residue associated primarily with lipid moieties. This is the first demonstration of the initial internalization of iron and its subsequent processing and secretion as a gelatinous residue containing phosphatidylethanolamine. Biotransformation is an important strategy initiated by microorganisms to circumvent toxic levels of metals in their environment (Silver & Misra 1988). Volatilization of mercury and insolubilization of lead enable bacteria to



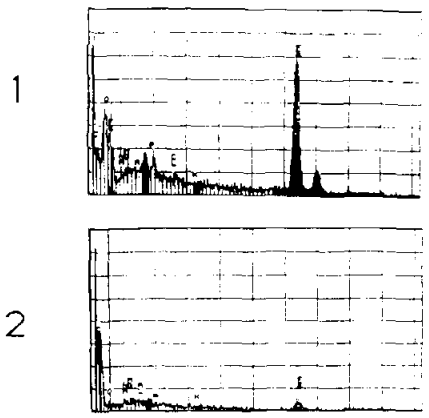


Figure 4. Scanning electron micrograph and energy dispersive X-ray microanalysis of the gelatinous residue. Note (1) irregular shaped bodies rich in iron and oxygen, and (2) elongated thread-like features rich in carbon.

(a) (b)

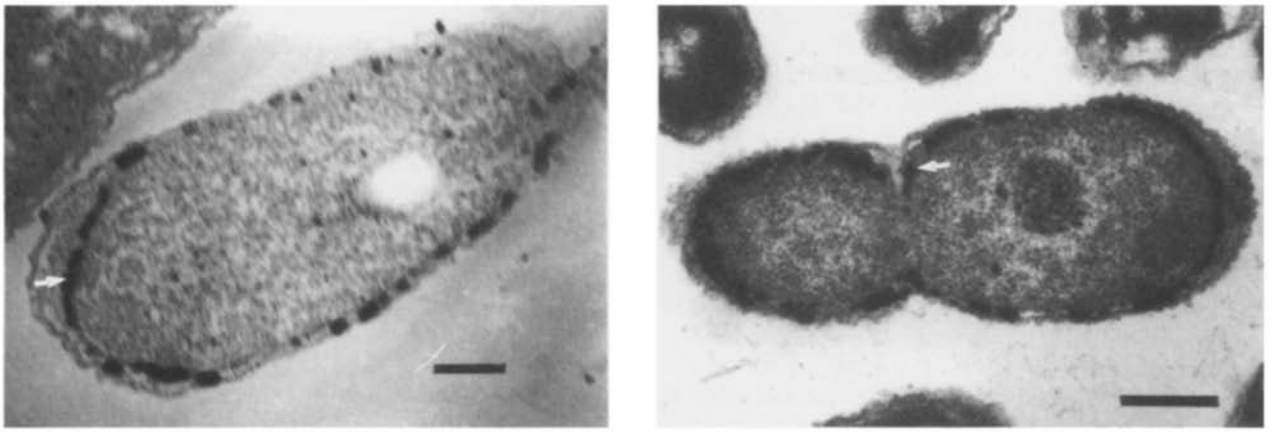


Figure 5. Transmission electron micrograph and EELS studies of iron stressed cells. (**a**) Ultrathin section of iron stressed *P. fluorescens* (uranyl acetate stained). Arrow indicates the iron deposits within the bacterial cell as examined by EELS. The bar represents 0.14 μm. (**b**) *P. fluorescens* with iron deposits in its dividing stage. (Uranyl acetate – lead citrate stained). The bar represents 0.33 μm.

tolerate toxic doses of these metals (Summers 1986, Al-Aoukaty *et al.* 1991). Reduction, oxidation and dealkylation of toxic metals into innocuous species are known to contribute to metal homeostasis in the microbial world (Silver & Misra 1988). We have recently demonstrated that the tolerance to elevated levels of calcium in *P. fluorescens* is mediated by the elaboration of calcite deposits (Anderson *et al.* 1992). In this instance iron(III), an essential element in trace amounts and toxic at elevated concentrations, is converted into hydrated oxide derivative(s). This biotransformation enables the bacterium to combat the inhibitory influence of the metal known to be involved in free radical generation (Halliwell & Gutteridge 1984). Numerous chemical forms of iron minerals have been tailored by different organisms to perform such biological tasks as storing iron, providing structural support and receiving magnetic signals. Ferritin is known to play a pivotal role in the mobilization of iron in eukaryotes and some prokaryotes. This biomolecule has several related protein subunits that surround a core of inorganic hydrated iron(III) oxide and is synthesized by some microbes in iron-rich media (Theil 1987, Mann & Perry 1991). It allows for the organization and utilization of soluble iron at concentrations and pH levels that may be toxic to the organisms. In this study the iron found in the soluble components of the bacterial cells may be associated with a similar protein moiety. As the organism was supplied with citrate complexed to iron, as the sole source of carbon, the microbe was forced to acquire a large excess of the trivalent metal in order to maintain a constant supply of energy. This situation may have been necessitated by the inability of bacteria to free the ligand from the metal extracellularly. The increment of pH of the spent fluid during growth would render the possibility of iron decomplexation via protonation quite unlikely. Citric acid is known to supply iron and also to act as an energy source in some pseudomonads (Harding & Royt 1990). Thus it is quite possible that the metal-bound tricarboxylic acid is internalized. Indeed the presence of iron in the soluble fraction of the microbial cells would support this postulation. This is further confirmed by the visualization of iron within the bacterial cells. Subsequent decomposition and/or localized acidification and/or reduction in the cells may enable the liberation of the ligand from the metal. While the ligand is then metabolized to promote growth, the metal is sequestered in the soluble cellular component prior to its secretion.

The elaboration of iron minerals is not an uncommon occurrence in the microbial world. The iron oxide Fe_3O_4 (magnetite) is synthesized by magnetotatic bacteria and plays a pivotal role in sensing geomagnetic gradients. Some iron reducing microbes are also known to form magnetite as a result of metabolic byproducts (Mann & Perry 1991). A magnetosome membrane that has a similar composition to the cell membranes appears to be the site of magnetite formation in magnetotactic bacteria. This enclosed vesicle provides an environment that allows for nucleation and growth of the biomineral. Following the uptake of the iron(III) from the environment, it is reduced and transported across the magnetosome. Within this vesicle precipitation of amorphous hydrated ferric oxide and formation of magnetite take place. The intracellular inclusions of Fe_3O_4 are localized in chains in close proximity to the inner surface of the cytoplasmic membrane. Morphologies of the magnetite appear to be dictated by the shapes of the membranes where the mineral is synthesized (Gorby *et al.* 1988, Blakemore & Blakemore 1990). The involvement of phospholipids in iron deposition has been demonstrated *in vitro* (Mann *et al.* 1986). In this instance, formation of the iron mineral was triggered upon the increase of the pH of the iron trapped lipid vesicle. Recently crystallization of iron

oxides in self assembled lipid tubules has also been demonstrated (Archibald & Mann 1993). In this study *P. fluorescens* secreted oxidized iron associated with lipid moieties, following its initial internalization in the soluble cellular fraction. Since iron(III) is deposited in association with lipid components, it is quite likely that these biomolecules may be providing an environment for the nucleation, deposition and eventual secretion of the trivalent metal. The constant intake of the rich dietary source of iron may limit the intracellular space where this element may be immobilized and also augment the vulnerability of the microbe to the free radical producing metal. Hence its removal as an insoluble deposit would allow for the maintenance of iron homeostasis. Thus since this trivalent is essential, it is first stored in the soluble cellular component of the cell; however, as its intake becomes excessive, the organism secretes the metal as an insoluble deposit. The phospholipid layers of the inner-membrane and/or of the innerleaflet of outer membrane would be an ideal site for the nucleation of the metal. The transmission electron micrograph would indicate such a possibility. The absence of lipopolysaccharide in the insoluble pellet would indicate that these moieties may not be participating in the detoxification of the metal. The fact that the cells with iron deposits were found in the mitotic stage and that the preconditioned cells grew rapidly in fresh iron medium clearly indicate the viability of the iron stressed *P. fluorescens*.

Since the solubility of iron(III) at the pH of the culture is extremely low, it is unlikely that the metal would stay in solution in the absence of any stabilizing molecule(s) following the consumption of citrate. Hence a gradual deposition of iron in the spent fluid concomittant with the utilization of citrate would have been observed. In this instance, it is tempting to speculate that the insolubilization of iron occurs only after the attainment of a certain critical concentration. It is also interesting to note that when this microbe was stressed with gallium, a metal that shares many similar features with iron(III), the immobilization of gallium was effected by soluble component(s) in the spent fluid (Al-Aoukaty *et al.* 1992). Indium, a trivalent metal whose toxicity has been linked to its ability to mimic iron(III), has been shown to be detoxified as an extracellular phosphorous containing derivative by the same microbe. In none of these stress conditions were the metals found to be associated with phospholipid(s) (Anderson & Appanna 1993).

In conclusion, this study demonstrates the attainment of iron(III) homeostasis in *P. fluorescens* via the elaboration of an exocellular lipid-rich amorphous iron mineral. It appears that the trivalent metal is initially internalized. Although the influence and the nature of the lipids involved in this iron regulation mechanism have to be further elucidated, it is tempting to speculate that these moieties may have a significant role in the synthesis and secretion of the iron mineral. The characterization of the soluble cellular iron component and the exocellular lipids and their role in the deposition of the iron mineral are currently in progress.

Acknowledgments

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