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Isolation of a membrane associated iron chelator from *Pseudomonas aeruginosa*

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A membrane associated iron chelator (MAIC) has been extracted with ethanol from the membranes of *Pseudomonas aeruginosa*, and isolated on thin-layer chromatograms. Also extracted from the membranes is the ferrated form of MAIC, FeMAIC. When cell-bound or in the complete ethanol extract of membranes, MAIC binds iron from exogenous iron sources forming FeMAIC. Methanol solutions of each compound exhibit similar absorption spectra with strong absorption in the ultraviolet, indicating the aromatic structure of the compounds. Colorimetric reactions reveal the presence of a phenolic moiety in these compounds. MAIC and FeMAIC are extracted from the membranes of cells grown in media supplemented with iron or in media containing significant trace levels of iron. Transport studies revealed that neither iron-fed nor iron-starved cells transport detectable levels of radiolabeled iron from exogenous iron sources, yet low amounts of ^{55}FeMAIC are extracted from the membranes of cells incubated with [^{55}Fe]ferric chelators. The MAIC may serve as an iron transporter in these cells, or may serve to bind iron following its transport into the cell via another mechanism.

Introduction

All microorganisms with the possible exception of the lactic acid bacteria require iron for growth [1]. As a component of the prosthetic group of many proteins, this element plays a critical role in numerous metabolic processes, including redox reactions, DNA synthesis, and nitrogen metabolism. Excess iron may be stored by some bacterial cells in the form of ferritin-like molecules [2,3].

Preceding the incorporation of iron into cellular protein, the iron must be acquired by the cell from the environment. The solubility of free iron at pH 7.0, 10^{-17} M, a concentration too low to sustain growth, mandates that microbes have a means to solubilize iron in order that it be available for utilization by the cell. Accordingly, bacteria developed high-affinity iron transport systems to acquire exogenous iron. These systems consist of low molecular weight iron chelators termed siderophores, and specific membrane receptors for iron transport into the cell. Following synthesis by bacteria growing under iron-deficient conditions, siderophores are secreted by the cells, bind iron in the surrounding media, and deliver the iron to the cell with the aid of the iron-regulated membrane receptors. Another class of siderophores consists of those that are not secreted by the cell but instead remain bound to the surface of the cell where iron binding takes place.

When grown in the presence of high levels of iron, bacteria synthesize neither siderophores nor iron-regulated membrane receptors, yet are able to acquire needed iron from insoluble ferric hydroxide compounds [4]. The specific steps of this

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low-affinity uptake of iron by bacteria have not been delineated.

The Gram-negative bacterium *Pseudomonas aeruginosa* reportedly produces two exogenous siderophores to acquire iron: pyochelin, an aqueous-insoluble phenolic compound [5,6], and pyoverdine, a yellow-green, water-soluble pigment [7]. It is also reported that *P. aeruginosa* acquires iron from citrate [8].

Evidence is presented here that *P. aeruginosa* contains an ethanol-extractable membrane associated iron chelator (MAIC). The finding that MAIC is isolated only from the membranes of cells grown in the presence of iron suggests that it is not a high-affinity transporter of iron, i.e., a siderophore of these cells. It is also shown that iron-fed cells do not transport detectable levels of iron bound to ethylenediaminedi(*o*-hydroxyphenylacetate) (EDDA) or to salicylate. However, in vivo, the membrane-associated chelator does bind radiolabeled iron from exogenous chelators, indicating that it may play a role in the transport of iron into these cells.

Materials and Methods

Bacteria and culture conditions

All experiments were done using *P. aeruginosa* ATCC 15692. For most experiments, cells were grown in the succinate synthetic medium (SSM) described by Meyer and Abdallah [9]. Cells were also grown in the above minimal media containing instead of succinate, 10 mM glucose (GSM) or sodium citrate (CSM), or in 0.25% casamino acids containing 0.2 mM $MgCl_2$ (pH 7.5) (CAA). When indicated, $^{55}FeCl_3$, 50 μCi, was added to 250 ml SSM. Cell growth was monitored by determining the absorbance at 600 nm using a Gilford Response UV/Vis Spectrophotometer. No attempt was made to deferrate media except when mentioned. In that case, prepared media was deferrated using Chelex 100 in a batch process. Culture flasks were routinely rinsed with 1 M HCl preceding final washing.

One liter aliquots of media were inoculated with 0.5 ml of a midlogarithmic phase culture, and the culture shaken at 150 rpm at 30 °C for 40 h in a New Brunswick rotatory shaker. During harvesting, cells were washed three times with 0.1 M 3-(*N*-morpholino)propanesulfonic acid (pH 7.2) (Mops buffer). When mentioned, cells were instead washed with 0.1 M Tris-HCl (pH 7.8) (Tris-HCl buffer), or with 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.2) (Hepes buffer).

Membrane preparation

Routinely, the cells of 2-liter growth media were washed and resuspended in 10 ml Mops buffer containing 2 mg each DNAase, RNAase, and lysozyme, and 1 mg each of $MgCl_2$ and phenylmethylsulfonyl fluoride. After 10 min incubation at room temperature, the suspension was passed one time through a precooled Carver pressure cell (Fred S. Carver Inc., Menomonee Falls, WI) at 20000 lb/in^2. The membranes were collected upon centrifugation at 147500 $\times g$ for 30 min, and washed twice in 0.1 M Mops buffer. When indicated, membranes were instead prepared using Hepes buffer or Tris-HCl buffer.

To separate the outer and inner membranes, the total membrane fraction was resuspended in Mops buffer to approx. 6 mg protein/ml, layered in 1 ml amounts on a sucrose density gradient consisting of 1 ml amounts of 58%, 64%, 70% and 75% sucrose (w/v), and centrifuged for 2 h at 175000 $\times g$ in a Beckman SW 50.1 rotor. The bands were collected, washed twice in Mops buffer, and frozen at −20 °C. This procedure is a modification of that of Hancock and Nikaido [10].

Analytical procedures and enzyme assays

The sugar 2-keto-3-deoxyoctonate (KDO) was assayed on membrane fractions according to the procedure of Weissbach and Hurwitz [11]. Membrane bound succinate dehydrogenase, malate dehydrogenase, and NADH oxidase were assayed following the method of Mizuno and Kageyama [12]. Protein concentration was determined according to the procedure of Lowry et al. [13] using bovine serum albumin as standard.

Pyochelin was extracted with ethyl acetate from acidified media [5]. Following evaporation of the ethyl acetate, the residue was dissolved in methanol and applied to a silica gel G thin-layer plate. The plate was developed in chloroform/ethanol/acetic acid (90 : 5 : 5, v/v) and examined for the presence of a yellow-green fluorescent spot of R_f 0.65.

Pyoverdine was extracted from spent media according to the procedure of Meyer and Abdallah [9]. Following extraction with 0.5 ml chloroform/phenol (1:1, v/w), and treatment of the organic phase with diethyl ether and water, the spectrum between 350 and 400 nm of the aqueous phase was determined using a Gilford UV/Vis Spectrophotometer.

Preparation of radiolabeled compounds

Radiolabeled iron was complexed to EDDA and to nitrilotriacetate (NTA) by adding acidic $^{55}FeCl_3$ to a solution of the chelator in a 30:1 chelator to iron ratio. [^{55}Fe]Iron salicylate was prepared in 0.05 M sodium acetate buffer according to the procedure of Ratledge and Marshall [14]. The final specific activity of each ferric chelate was approximately 4 μCi/μmol. ^{55}Fe-pyoverdine was prepared by adding radiolabeled iron to pyoverdine purified from spent medium according to the procedure of Meyer and Abdallah [9].

Extraction, radiolabeling, and quantitation of the MAIC

6 ml of ethanol were added to the total membrane fraction isolated from 1 liter of cells. The suspension was mixed and incubated at room temperature for 1 h with occasional resuspension. Following centrifugation of the suspension at $35\,000 \times g$ for 30 min, the ethanol extract was removed and treated as indicated in the text. A similar extraction procedure was carried out using whole cells. In some cases, whole cells were incubated with radiolabeled compound for 30 min, after which time the cells were washed three times with Mops buffer. Membranes were then isolated from these cells and ^{55}FeMAIC extracted with ethanol.

Thin-layer chromatography (silica gel G) of ethanol extracts were routinely done using chloroform/ethanol/acetic acid (90:5:5, v/v) (solvent A). Some chromatograms were instead developed in petroleum ether/*n*-butanol/ethyl acetate (2:3:3, v/v) (solvent B). Bands were scraped from the plates, and resuspended in methanol. The absorption spectra of the compounds were obtained using a Gilford Response UV/Vis Spectrophotometer. Peak locations and intensities were identified by the spectrophotometer. To further separate the individual compounds, an ethanol extract of membranes was applied to a column (1 × 36 cm) of Sephadex LH-20 (Pharmacia) equilibrated with methanol.

Radiolabeling of MAIC was routinely done using $^{55}FeCl_3$ (New England Nuclear, 23.85 mCi/mg). When mentioned, MAIC was instead radiolabeled using ^{55}FeEDDA, ^{55}FeNTA, [^{55}Fe] iron salicylate, or ^{55}Fe-pyoverdine. The location of any radiolabeled compound on the thin-layer chromatograms was detected by putting 1 cm^2 scrapings of the matrix from the plate into Optifluor scintillation fluid (Packard). ^{55}FeMAIC was localized on the plate by its purple color. The samples were counted in a Packard 300 CD liquid scintillation counter.

Colorimetric determinations

To determine if FeMAIC contains phenolic moieties, the compound was scraped from a thin-layer plate (solvent B) and eluted with methanol. The compound in solution was acid hydrolyzed with HCl to 1 M and rechromatographed. A 1% $FeCl_3$/1% potassium ferricyanide solution was sprayed on the plate to determine any presence of phenolic moieties [15]. Acid hydrolyzed and unhydrolyzed FeMAIC were also assayed according to the Arnow method [16] to detect catechols, and according to the Csaky procedure [17] to detect any hydroxamate groups. In carrying out the Csaky procedure, the samples were acid hydrolyzed for 3 h at 100°C followed by autoclaving at 121°C for 1 h [18].

Transport assays

Cells were grown in SSM containing 64 μM $FeCl_3$ and in deferrated SSM to mid logarithmic phase and to early stationary phase. Following three washes and resuspension in Mops buffer, cells were equilibrated at 30°C for 20 min in buffer or in buffer containing 6.6 mM succinate. Radiolabeled iron, 3.8 ng, complexed to chelator (EDDA or salicylate) was then added to the cell suspension. Aliquots were withdrawn at set time intervals and added to 5 ml ice-cold Mops buffer on a Metricel GA-6 filter, porosity 0.45 μm. Following filtration, the cells on the filter were washed twice with 3 ml buffer as above. The filter was transferred to a liquid scintillation vial, Optifluor added, and the sample counted.

Results

Isolation of an iron-binding and an iron-containing compound from cell membranes

The ultraviolet and visible spectrum of the ethanol extract of isolated membranes of cells grown in SSM is shown in Fig. 1. Upon chromatographing the ethanol extract of isolated membranes in solvent A, a yellow band of R_f 0.60 and a purple band of R_f 0.77 were evident. Also visible on these chromatograms was a brown band of R_f 0.85. The yellow and purple bands were scraped from the plates, eluted with methanol, and the ultraviolet and visible spectra determined (Fig. 2). It is seen that each compound absorbs strongly in the ultraviolet, and that the purple band absorbs more strongly at 257.5 nm than does the yellow band. Visible absorptions for both compounds were relatively weak. There was slight difference between the visible spectra of the two compounds even when the full scale absorbance was set to 0.5. Characteristic of the ultraviolet spectra of the yellow and purple bands and seen in the complete ethanol extract is a doublet at 315 and 327.5 nm. Here, the yellow band is referred to as the membrane associated iron chelator (MAIC). As shown below, the MAIC binds iron forming the purple compound, FeMAIC.

Membranes from stationary phase succinate grown cells were extracted with ethanol for varying periods of time to determine the time required for maximum extraction of the compounds. Spectral analyses at given time intervals revealed the characteristic spectrum seen in Fig. 1. Extraction of the two compounds from the membranes of succinate grown cells was completed within 30–60 min as detected by the lack of increase in absorbance at 327.5 nm.

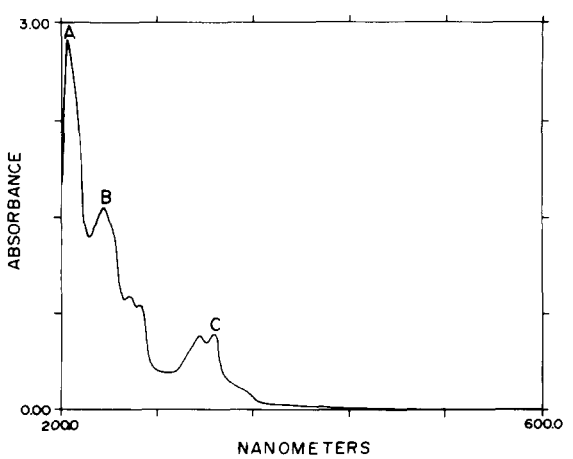

Fig. 1. Ultraviolet and visible spectrum of the ethanol extract of membranes, in methanol. Peak A, 204.5 nm; Peak B, 235.5 nm; Peak C, 327.5 nm.

Properties of FeMAIC

Addition of HCl to 1 M, to a methanol solution of FeMAIC resulted in a change of color from purple to yellow. Chromatography of the acid hydrolysate resulted in two yellow spots, one of R_f 0.86 and one of R_f 0.41 (solvent A). Lack of a purple band suggested that deferration occurred upon acid hydrolysis. Spraying this plate with a 1% $FeCl_3$/1% potassium ferricyanide solution caused the faster migrating band to turn blue. Analysis of FeMAIC by the Arnow procedure and by the modified Csaky procedure were negative.

In addition to being soluble in ethanol and methanol, the two compounds were also soluble in chloroform, ethyl acetate, *n*-butanol, benzene, and acetone. They were slightly soluble in ether, and not soluble in aqueous solution.

Radiolabeling of MAIC to form ^{55}FeMAIC

$^{55}FeCl_3$ (0.2 μCi) was added to the ethanol extract of membranes. Following incubation with stirring for 10 min, the extract was applied to a thin-layer plate. Upon development in solvent A, the chromatogram revealed the incorporation of label into the purple spot (Fig. 3), and the concomitant loss of MAIC. Under these conditions, no radiolabeled iron was detected in the brown band, also an iron-containing band as revealed by its radioactivity when extracted from membranes of cells grown in the presence of $^{55}FeCl_3$, conditions under which the purple spot was also radiolabeled. In another experiment, the absorbance at 327.5 nm of FeMAIC increased from 0.824 to 4.758 upon addition of $8.26 \cdot 10^{-6}$ mg $FeCl_3$ to an ethanol extract of membranes. No MAIC was detected in the extract to which the iron was added. Upon adding $^{55}FeCl_3$ to the ethanol extract of membranes prepared using Hepes buffer or Tris-HCl buffer, ^{55}FeMAIC was again isolated on thin-layer chromatograms, indicating that the iron is not nonspecifically binding to a cellular or

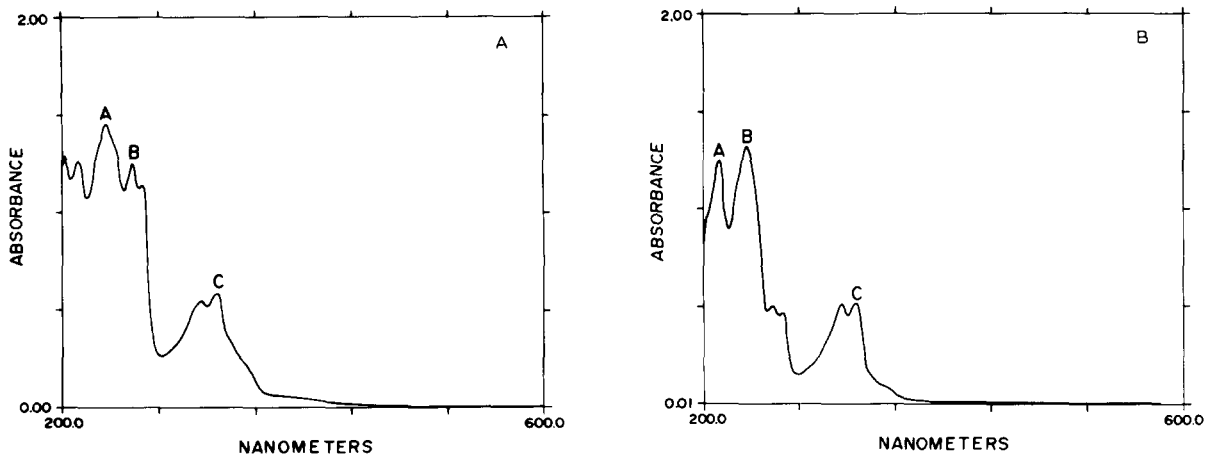

Fig. 2. Ultraviolet and visible spectra of two bands resolved on a thin-layer chromatogram, a chromatogram to which an ethanol extract of membranes had been applied. (A) The purple band (FeMAIC). Peak A, 236 nm; Peak B, 257.5 nm; Peak C, 327.5 nm. (B) The yellow band (MAIC). Peak A, 213.5 nm; Peak B, 235.5 nm; Peak C, 327.5 nm.

membrane component extracted due to the presence of Mops buffer. It was also shown that an ethanol extract of membranes chelated iron from

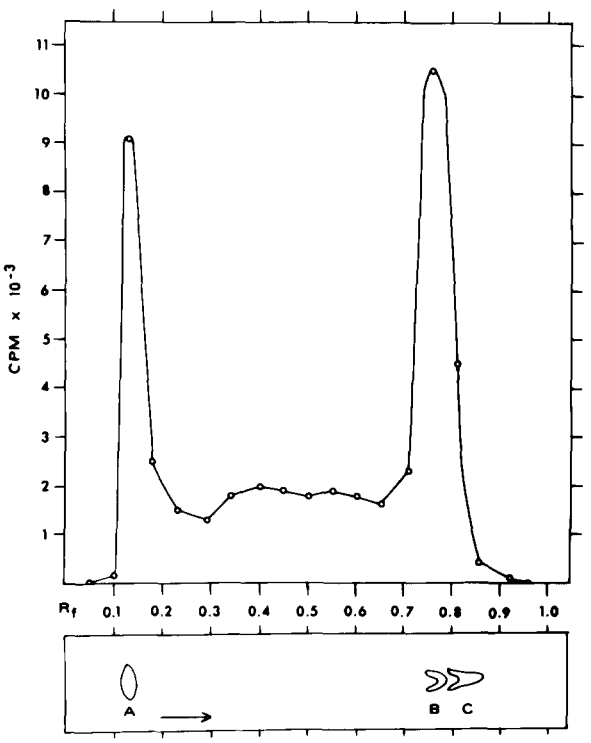

Fig. 3. Distribution of radiolabeled iron on a thin-layer chromatogram. $^{55}FeCl_3$ was added to an ethanol extract of membranes and the sample chromatographed. A, Origin; B, purple band; C, brown band.

[^{55}Fe]iron salicylate, ^{55}FeNTA, ^{55}FeEDDA, and ^{55}Fe-pyoverdine.

It was of interest to determine the extraction procedure which resulted in the maximum yield of FeMAIC. To do this, $^{55}FeCl_3$ (0.2 μCi) was added to the ethanol extract of whole cells and to the ethanol extract of isolated membranes. Also, whole cells and membranes were incubated with $^{55}FeCl_2$ (0.2 μCi) for 30 min prior to extraction of ethanol soluble material. Iron incorporated into MAIC was determined upon chromatographing the extracts. It is seen in Table I that maximum recovery of $^{55}FeCl_3$ occurred upon adding $^{55}FeCl_3$ to the ethanole xtract of isolated membranes. Addition of $^{55}FeCl_3$ to the membranes and to whole cells prior to ethanol extraction resulted in a lesser yield of recoverable ^{55}FeMAIC (Table I).

TABLE I

INCORPORATION OF ^{55}Fe INTO MAIC

$^{55}FeCl_3$ added to: [a]	dpm in ^{55}FeMAIC
Ethanol extract	
of whole cells	2590
of membranes	8400
Whole cells [b]	920
Membranes [b]	620

[a] Washed cells of 250 ml culture media were used in each extraction.

[b] Whole cells or membranes were incubated with 0.2 μCi $^{55}FeCl_3$ after which time the sample was extracted with ethanol.

Incubation of whole cells with ^{55}FeEDDA or [^{55}Fe]iron salicylate followed by ethanol extraction of the isolated membranes resulted in relatively low yields of ^{55}FeMAIC on thin-layer chromatograms. Upon incubating cells with ^{55}FeEDDA, 250 dpm were recovered in ^{55}FeMAIC, as compared with 875 dpm from membranes of cells incubated with [^{55}Fe]iron salicylate.

To determine if isolated MAIC chelated iron in the absence of other factors in the ethanol extract, MAIC was eluted from the gel matrix with methanol and incubated with $^{55}FeCl_3$ (0.2 μCi). Upon chromatographing the sample, MAIC was recovered, but no ^{55}FeMAIC was detected. Also, MAIC on a thin-layer plate did not turn red upon spraying with 0.1 M $FeCl_3$.

Sephadex LH-20 chromatography

A concentrated ethanol extract of membranes was applied to a Sephadex LH-20 column equilibrated with methanol. Three peaks were visually evident, a brown peak followed by two yellow peaks. Each of the three peak fractions was collected, condensed, and divided in half. $^{55}FeCl_3$ (0.2 μCi) was added to half of each fraction, and the six samples were applied to a thin-layer chromatogram. The material of peak I did not migrate. Peak II contained FeMAIC as evidenced by a purple band. However, radiolabeled iron was not incorporated into the FeMAIC: the added iron remained at the application spot. Chromatography of peak II also resulted in the appearance of the brown spot mentioned earlier. Most of the material in peak III remained at the application spot, yet material with slight yellow fluorescence with an R_f of about 0.5 was noted in these lanes. All radiolabeled iron added to peak III was recovered at the application spot. These results show that isolated fractions of the ethanol extract do not bind iron.

Extraction of MAIC from cells grown in different media

To determine if MAIC is present in cells grown in media other than succinate-containing media, *P. aeruginosa* was grown in CAA media, citrate-containing media, and glucose-containing media. $FeCl_3$ (30 μM) was added to one aliquot of each type of media. Membranes from cells harvested during the early stationary phase of growth were extracted with ethanol for 1 h. $^{55}FeCl_3$ (0.2 μCi) was added to each ethanol extract and the amount of ^{55}FeMAIC determined upon developing the extracts in solvent A. It is seen in Table II that the ethanol extract of cells grown in CAA and GSM media unsupplemented with iron did not bind iron. The presence of iron in these two types of media did result in ethanol-extractable iron-chelating activity from the membranes. Growth in CSM with and without added iron resulted in production of MAIC. It is also seen in Table II that less MAIC is extracted from the membranes of cells grown in deferrated SSM than from membranes of cells grown in unsupplemented SSM and in SSM containing 30 μM $FeCl_3$.

TABLE II

IRON-BINDING CAPACITY OF ETHANOL EXTRACTS OF MEMBRANES OF EARLY STATIONARY PHASE CELLS

Growth media	dpm per 250 *A* units of cells [a]
CAA	0
CAA + $FeCl_3$ [b]	29547
CSM	3355
CSM + $FeCl_3$ [b]	6675
GSM	0
GSM + $FeCl_3$ [b]	10933
SSM (deferrated)	890
SSM	40710
SSM + $FeCl_3$ [b]	38040

[a] dpm in ^{55}FeMAIC recovered on thin-layer chromatogram.
[b] 30 μM $FeCl_3$.

TABLE III

CONSTITUENTS OF MEMBRANE FRACTIONS ISOLATED ON A SUCROSE DENSITY GRADIENT

Fraction	MDH [a]	SDH [a]	NADH oxidase [b]	KDO [c]
1	160	4.0	463	7.2
2	57	0.95	97	8.3
3	12	0.20	48	17.0
4	52	0.40	50	13.4

[a] MDH, malate dehydrogenase; SDH, succinate dehydrogenase. Activity is expressed as nmol of dichlorophenolindophenol reduced per min per mg of protein.
[b] Activity is expressed as nmol NADH oxidized per min per mg of protein.
[c] KDO, 2-keto-3-deoxyoctonate. Amount is expressed as μg per mg protein.

TABLE IV

LOCATION OF MAIC ON ISOLATED MEMBRANE FRACTIONS

Fraction	dpm in ^{55}FeMAIC [a]
1 [b]	3280
2	680
3	0
4	0

[a] $^{55}FeCl_3$ was added to the ethanol extract of each membrane fraction. dpm in ^{55}FeMAIC was determined on thin-layer chromatograms.

[b] Fraction refers to membrane fractions isolated on a sucrose density gradient (Table III).

Localization of MAIC

The outer and inner membranes of cells grown in SSM were separated upon breaking cells using a pressure cell. Sucrose density gradients of the washed membrane fractions revealed four bands. The distribution of marker enzyme activity and 2-keto-3-deoxyoctonate (KDO) revealed that band 1, which was visibly red, consisted of the inner membrane, and that band 3 was the purest outer membrane fraction (Table III). Each of the bands isolated was extracted with ethanol for 1 h. $^{55}FeCl_3$ (0.2 μCi) was added to each extract and the amount of ^{55}FeMAIC determined via thin-layer chromatography. The greatest amount of radiolabeled iron was incorporated into the ethanol extract of band 1, the inner membrane. The outer membrane fraction failed to incorporate label (Table IV).

Transport of radiolabeled iron by whole cells

Neither pyoverdine nor pyochelin was detected in the spent media of cells grown in deferrated SSM or in SSM containing 64 μM $FeCl_3$. This allowed examination of transport into washed cells without interference by residual levels of exogenous siderophores. Initial attempts to study radiolabeled iron transport into whole washed cells using $^{55}FeCl_3$ were unsuccessful for the insoluble iron complexes that formed at physiological pH were nonfilterable, and hence, erratic trapping of radiolabeled iron by the filter occurred. Therefore, iron complexed to EDDA and to salicylate was used. Neither iron-starved nor iron-fed cells transported detectable levels of radiolabeled iron from either chelator over a 20 min time period. The addition of succinate to resuspended cells failed to stimulate the uptake of iron.

Discussion

Evidence is presented that *P. aeruginosa* contains an inner membrane associated iron chelator. Called MAIC, this compound chelates iron when bound to the membrane or when in the presence of a full ethanol extract of whole cells or of the membrane fraction of these cells. The iron-containing form of MAIC is termed FeMAIC. Failure of isolated MAIC to bind added iron suggests that other ethanol extractable material is needed to stabilize MAIC for iron binding or perhaps to interact with MAIC to promote its binding of iron.

Absence of a positive Csaky reaction suggests that MAIC does not contain hydroxamate groups. The blue color observed upon spraying acid hydrolyzed FeMAIC with $FeCl_3$/potassium ferricyanide solution indicates the presence of a phenolic moiety in the compound [15]. An intense ultraviolet absorption like that exhibited by both MAIC and its ferri form in methanol is known to result from aromatic rings within ligand structures [19]. Absorption specific to a metal-ligand complex are of two classes [20,21]. First, absorptions in the visible are attributed to electrons associated mainly with the metal itself; these absorptions always exist but are usually extremely weak. Second, absorptions in the ultraviolet are strong and are attributed to electrons that are transferred between the ligand and the metal. Exceptions to this have been observed where the second class (transfer type) absorptions are shifted from the ultraviolet to the visible region when hydroxamates or catechols are involved in a metal-ligand complex [19]. The ferri form of MAIC does have a purple coloration or blue-green absorption in the visible when on the gel matrix, with and without the chloroform solvent. In methanol, however, no comparable blue-green absorption is observed. It is well known that different solvents can complex with metals and ligands to varying degrees [22]. It is also known that absorption peaks may shift in position and intensity due to solvation effects and interactions with a solid surface

[23]. The purple coloration on the gel may be due to these effects. The virtually nondetectable visible absorption for the ferri form in methanol is consistent with the first class of metal-ligand absorptions. While the extremely small visible absorption is not useful for ferri form identification at the concentrations used here, the low absorption would be consistent with a lack of catechols in MAIC which would require a concomitant visible shifted absorption of the second class. The strong ligand-metal electron transfer ultraviolet peak at 257.5 nm for the ferri form in methanol may be used for identification. The ferri-free MAIC ultraviolet absorption peaks persist together with the additional ferri form peak.

More MAIC was extracted with ethanol from isolated membranes than from whole cells. If the MAIC is located on the cytoplasmic side of the inner membrane, the presence of inverted vesicles may facilitate extraction of ethanol soluble material. The low recovery of ^{55}FeMAIC from whole cells incubated with $^{55}FeCl_3$ prior to extraction with ethanol may be due to utilization of the iron by the cell preceding extraction with ethanol. The low recovery of ^{55}FeMAIC from membranes as well as from whole cells incubated with $^{55}FeCl_3$ prior to extraction with ethanol may instead indicate that iron serves to hold the ferrated compound to the membrane, resulting in the incomplete extraction of this compound. At this time it is not certain if all of the FeMAIC observed on thin-layer chromatograms of an ethanol extract of membranes to which $^{55}FeCl_3$ was not added is the ferrated compound that was actually on the membrane at the time of extraction, or if all or a portion of the FeMAIC was formed during the extraction procedure. The possibility exists that trace levels of iron bound to glassware and to the silica gel matrix may be sequestered by MAIC during the isolation procedure.

The concentration of iron in the media effects the amount of MAIC extracted from the membranes of cells, and in general, less MAIC is extracted from cells grown under iron-deficient conditions than from iron-fed cells. Membranes of cells grown in CSM unsupplemented with iron did yield more MAIC than did membranes of cells grown in unsupplemented GSM or CAA. This may be explained by the fact that citrate serves as an iron transporter of these cells [8]. Hence, by chelating trace levels of iron in the media, citrate is making more exogenous iron available to the cells than is available in GSM or in CAA media. However, the addition of iron to CSM did result in a higher yield of MAIC than was obtained from cells grown in unsupplemented CSM. The presence of MAIC on the membranes of cells grown in SSM, CSM, GSM, and CAA media indicates that the iron chelator is not a unique property of cells grown on any one growth media.

The cytoplasmic membrane location of the isolated iron chelator, its insolubility in aqueous solution, and the ease with which it is extracted from the membrane with ethanol make it similar to the mycobactins of the mycobacteria [24]. The mycobactins, however, are isolated from the membranes of cells grown in iron-poor media [14]. It has been suggested that the mycobactins are iron storage molecules and not iron transporters [25]. Pathogenic *Neisseria meningitidis* also possesses a cell-associated iron chelator as evidenced by the capacity of this organism to sequester iron directly from ferritransferrin [26]. This transporter constitutes a high affinity system for it is found only in iron-starved cells.

To determine if a membrane bound iron transporter exists in *P. aeruginosa,* iron transport studies were done using iron-starved and iron-fed cells, neither of which produced pyochelin or pyroverdine. Initial transport studies were done using $^{55}FeCl_3$, for it has been reported that *P. aeruginosa* sequesters iron from radiolabeled $FeCl_3$ [27,28]. The methodology employed in those studies, i.e., addition of $^{59}FeCl_3$ to spent media of iron-starved cells, allows for transfer of added iron to exogenous siderophore preceding transport into the cell. Transport studies were performed here in the absence of exogenous siderophores. Hence, solubilization of ferric complexes at physiological pH did not occur, and such complexes consequently prevented accurate determination of any uptake of radiolabeled iron from $FeCl_3$. Transport studies were therefore performed using radiolabeled iron complexed to EDDA and to salicylate, the latter chelator being that one used to demonstrate iron acquisition by *Mycobacterium smegmatis* via membrane-bound mycobactin [14]. It was first shown that iron complexed to each

chelator was sequestered by MAIC in an ethanol-extract of membranes. Upon carrying out the described transport procedure using either ^{55}FeEDDA or [^{55}Fe]iron salicylate, no detectable amount of iron was taken up by iron-fed or iron-starved cells. However, to further determine if the MAIC on membranes of cells can bind iron from an external exogenous source, whole cells were incubated with ^{55}FeEDDA and with [^{55}Fe]iron salicylate for 30 min, after which time membranes were isolated and extracted with ethanol. Small amounts of ^{55}FeMAIC were extracted from the membranes under these conditions. However, the possibility exists that not all the ^{55}FeMAIC formed under these conditions was extracted from the membranes, for it was seen that the ferrated form of MAIC is less easily recovered from the membrane than is ferri-free MAIC. That external iron is sequestered by MAIC in whole cells was also seen upon extracting ^{55}FeMAIC from whole cells incubated with $^{55}FeCl_3$. Hence, MAIC does sequester iron from exogenous sources. If MAIC is directly involved in the transport of iron into the cells is not known at this time. Emery [29] has recently hypothesized that in addition to high-affinity iron transport systems consisting of siderophores and specific membrane receptors, microorganisms also contain a second system of iron transport, a reductive system which is membrane bound and nonspecific in its recognition of iron donor [29]. Perhaps MAIC is a candidate for such a transport system. The possibility also exists that MAIC serves to chelate iron subsequent to transport via a different mechanism. It was shown that MAIC in the ethanol extract of membranes does sequester iron from pyoverdine, the most effective exogenous siderophore of *P. aeruginosa* [30]. MAIC may serve instead as an iron storage compound or in an iron-requiring metabolic reaction following transport into the cell. Experiments are presently underway to further delineate a role of MAIC in iron metabolism of *P. aeruginosa.*

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