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Effects of iron(III) analogs on growth and pseudobactin synthesis in a chromium-tolerant *Pseudomonas* isolate

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Summary. The growth and siderophore production of a fluorescent *Pseudomonas* species isolated from soil contaminated with chromium was found to be influenced by the presence of trivalent cations. Overproduction of pseudobactin occurred when the isolate was grown in media containing 1 mM Cr(III) under iron-limited conditions but not when Fe(III) was added at 10 μM. Pseudobactin synthesis was derepressed in iron-limited cultures containing 1 mM Sc(III) or Y(III), examples of group III-B elements. We found that Al(III), Ga(III) or In(III), representative metals from group III-A, repressed synthesis of pseudobactin under iron-deficient conditions. Analogs of Fe(III) were found to inhibit growth of the *Pseudomonas* isolate in iron-limited media and the trivalent metals listed in order of decreasing toxicity were as follows: Ga > In > Sc > Cr > Y > Al. The inhibition of growth by 1 mM In(III), Sc(III) and Ga(III) was greater during iron-limited growth than in media containing 10 μM Fe(III). These data show that, although the metal analogs of Fe(III) have similar chemical and physical characteristics, the physiological response of the fluorescent pseudomonad when grown in the presence of these metals varied markedly.

Key words: Pseudobactin siderophore - Iron metabolism - Chromium - Gallium - Scandium

Introduction

Uptake of iron by microorganisms may be dependent on a specific high-affinity iron acquisition system (Hider 1984; Neilands 1984). Components of this high-affinity system include release of siderophores into the extracellular environment to sequester Fe(III) and the deployment of cognate membrane receptor proteins. Alternatively, most bacteria have a low-affinity Fe(III) uptake system which functions when environmental iron is present in high concentration. Siderophores are not involved in the low-affinity iron pathway (Hider 1984; Neilands 1984).

Metals that mimic Fe(III) may be expected to compete with iron for ligands in vivo and in the mediation of low-affinity Fe(III) assimilation. Either of these activities involving competition between metals would result in perturbation of normal physiological functions (Hughes and Poole 1989). However, metal analogs of siderophores are experimentally useful in biochemical and physiological investigations involving the transport of metal chelates. Ga(III) complexes with siderophore ligands or with citrate were used to investigate reductive mechanisms of iron assimilation in *Mycelia sterilia* and *Ustilago sphaerogena* (Adjimani and Emery 1987; Emery 1987) and *Rhodopseudomonas sphaeroides* (Moody and Dailey 1985). Siderophores in which Fe(III) has been replaced with Cr(III), Ga(III) or Al(III) have proved useful in probing specificity of iron transport systems in *Neurospora crassa* (Winkelmann et al. 1973) and *U. sphaerogena* (Emery 1971; Emery and Hoffer 1980; Leong et al. 1974). Complexes of Sc(III) and In(III) with enterobactin have been shown to exert bacteriostatic effects on pathogenic serotypes of *Escherichia coli* and *Klebsiella pneumoniae* (Rogers 1987).

We report here the physiological response of a fluorescent pseudomonad soil isolate, in terms of growth and siderophore synthesis, when grown in the presence of various trivalent metal analogs of Fe(III). We found that the synthesis of pseudobactin, the characteristic yellow-green fluorescent siderophore formed by certain *Pseudomonas* sp. (Meyer et al. 1987; Buyer et al. 1990; Teintze et al. 1981), was derepressed in iron-limited cultures supplemented with high concentrations of Cr(III), Sc(III), and Y(III), but was repressed in the presence of Al(III), Ga(III), or In(III). Furthermore, metal mimicry exerted differential effects on growth as a function of the iron nutritional status of the pseudomonad isolate.

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Materials and methods

Bacterial strain. The bacterial strain, designated LC-11, used in this study was isolated from chromium-contaminated soil at the Los Alamos National Laboratory (Los Alamos NM). The isolate was identified as a fluorescent *Pseudomonas* sp. and the metabolic characteristics of this strain are to be published elsewhere. The culture was maintained on slants consisting of plate count agar (Difco Laboratories, Detroit MI).

Media. A chemically defined medium was developed to support growth of the isolate in experimental tests with high levels of trivalent metal salts and for maintenance of conditions of low-iron availability. For 1 l medium, 7 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g sodium citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, and 15 g 1,4-piperazinediethanesulfonic acid (Pipes; Sigma Chemical Co., St. Louis MO) were dissolved in 980 ml deionized water. The pH of the medium was adjusted to 7.0 using NaOH. After autoclaving, the following sterile stock solutions were added: 10 ml 20% glucose, 1 ml trace element solution, 7.5 ml vitamin solution, and 1 ml Fe(III) solution when appropriate. Trace element solution consisted of 2 mg $MnSO_4 \cdot H_2O$, 1 mg H_3BO_3, 1 mg $CuSO_4 \cdot 5H_2O$, 2 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 mg $ZnSO_4$, 20 mg $CaCl_2 \cdot 2H_2O$, 50 mg EDTA and 100 ml deionized water. The Fe(III) solution was prepared by adding 0.3 g ferric citrate to 100 ml deionized water. The vitamin solution was composed of 0.2 g pyridoxine·HCl, 0.2 g nicotinic acid, 0.2 g nicotinamide, 0.4 g thiamin·HCl, 8 mg biotin, and 1 l deionized water. The glucose was autoclaved separately and the trace element, Fe(III), and vitamin solutions were filter-sterilized. Solutions of $CrCl_3$, $AlCl_3$, $InCl_3 \cdot 4H_2O$, $GaCl_3$, YCl_3, or $ScCl_3 \cdot H_2O$ (all obtained from Aldrich Chemical Co., Milwaukee WI) were filter-sterilized and added to culture media as indicated. For solid-plating media, 15 g noble agar (Difco) was added.

Growth response in the presence of metals. The inoculum for various experiments was grown in 5 ml iron-deficient defined medium in 16·125-mm screw-capped test tubes for 18 h on a tissue culture roller drum at 28° C; 2 ml inoculum was introduced into 100 ml metal-supplemented medium in a 500-ml conical flask. The culture flasks were incubated at 28° C with gyratory shaking at 200 rpm. At intervals, samples were withdrawn and viable bacteria were enumerated using standard microbiological techniques with plate count agar used to support growth.

Pseudobactin isolation. To verify the formation of pseudobactin by the pseudomonad isolate grown under various conditions of metal stress, the siderophore was isolated from cultures using a procedure modified from Teintze et al. (1981) and Fekete et al. (1989). After incubation for five days, cultures were centrifuged at $12000 \times g$ for 20 min. The yellow-green fluorescent supernatant fluids were collected and $FeSO_4 \cdot 7H_2O$, at 2 mg/ml culture volume, was added. The ferrated solution was agitated vigorously on a rotary shaker at 250 rpm for 4 h, then saturated with ammonium sulfate. Benzyl alcohol (0.2 vol.) was added and this slurry was agitated for 2 h at 250 rpm before being centrifuged at $8000 \times g$ for 20 min. The red-orange organic layer containing siderophore was carefully drawn off with a pipette. Pseudobactin was partitioned into water from the organic phase by addition of 4 vol. diethyl ether and 0.1 vol. water. The aqueous extracts were washed with diethyl ether to remove benzyl alcohol and absorption spectra of ferric pseudobactin were recorded with a Beckman model 25 spectrophotometer.

Analytical procedures. Chrome azurol S (Schwyn and Neilands 1987) blue agar plates, composed of the chemically defined basal medium, were used to screen the siderophore activity of the isolate. Formation of siderophore was determined by the procedure of Buyer et al. (1990). Ferric pseudobactin concentration in culture supernatant fluids was estimated by measuring the absorbance at 400 nm. The molar absorption coefficient of $2 \times 10^4\ M^{-1}\ cm^{-1}$ was used to determine the quantity of pseudobactin present. Generation times of *Pseudomonas* strain LC-11 in metal-amended cultures were calculated from plotted data and from linear regression analysis (Koch 1981).

Results

Characteristics of Pseudomonas *sp. strain LC-11*

The isolate LC-11 was found to form high levels of siderophore as evidenced by large orange halos surrounding colonial formation on chrome azural S plating medium. Upon plating LC-11 on low-iron medium, an extensive water-soluble, fluorescent yellow-green pigment was produced after 2–3 days of growth. The Gram-negative, oxidase-positive motile bacillus was identified as a fluorescent *Pseudomonas* sp.

LC-11 tolerated very high levels of Cr(III), i.e. the strain grew on solid-plating media amended with 10 mM $CrCl_3$. The amount of fluorescent pigment, or chromophoric pseudobactin (pyoverdin; Meyer et al. 1987), was observed to be proportional to the level of Cr(III) added to iron-free solid media. When Fe(III) was added in conjunction with 10 mM Cr(III) to-solid-plating cultures of LC-11, the pigment was also produced.

Growth and viability of strain LC-11 in metal-supplemented media

Growth and viability of LC-11 in liquid cultures supplemented with Fe(III), Cr(III), Al(III), In(III), Ga(III), Y(III) or Sc(III) was determined using the standard viable plate count technique. In the presence or absence of iron in media supplemented with Cr(III), LC-11 showed no apparent lag phase and rates of growth were identical (Fig. 1a and Table 1). However, Cr(III)-supplemented cultures showed a substantial diminution of maximum viable cell numbers at the stationary phase of growth in comparison to Cr(III)-untreated controls (Table 1). Marked differences in growth and viability of iron-deficient and iron-replete cells of LC-11 were apparent when the strain was grown with the group III-A elements, Al(III), In(III), or Ga(III) (Fig. 1b and Table 1). Ga(III) at a concentration of 1 mM was found to be the most active bacteriostatically to the organism when iron was omitted from the growth medium. Iron addition spared Ga(III) growth inhibition only after a growth lag of 46 h; the stationary phase was not reached until after 72 h of incubation due to a substantially decreased rate of growth. In the absence of added iron, the concentration of Ga(III) had to be decreased to 0.5 mM before growth ensued after a growth lag of 46 h (Fig. 1b and Table 1). With 0.5 mM Ga(III), LC-11 had a generation time of 580 min and the stationary phase of growth was not reached until 120 h of incubation.

Similarly, the addition of Fe(III) significantly relieved inhibition attributed to In(III) so that growth in

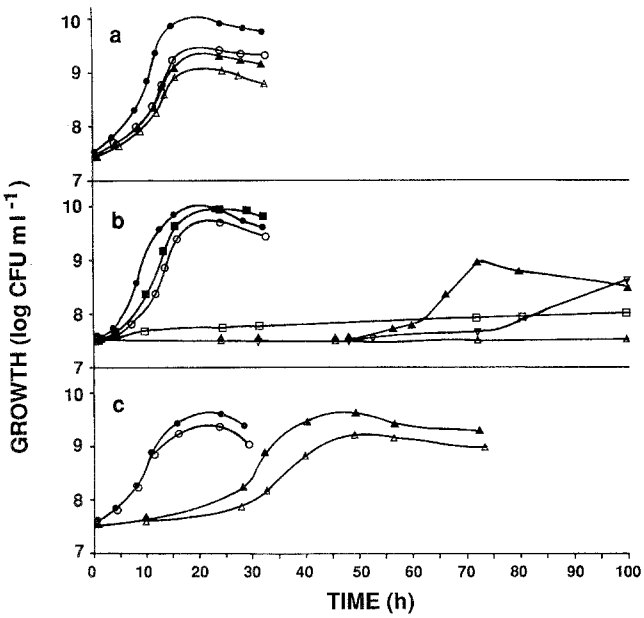

Fig. 1. Growth of *Pseudomonas* strain LC-11 in liquid medium with added iron (closed symbols) and without added iron (open symbols). **a** Cells grown in control untreated media (●, ○) or in media supplemented with 1 mM Cr(III) (▲, △); **b** cells grown in media supplemented with 1 mM Al(III) (●, ○), 1 mM In(III) (■, □), 1 mM Ga(III) (▲, △), or 0.5 mM Ga(III) (▽); **c** cells grown in media supplemented with 1 mM concentrations of Y(III) (●, ○) or Sc(III) (▲, △). Mean values of duplicate experiments are plotted; CFU = colony-forming units

Table 1. Generation times and reduction in growth yield of *Pseudomonas* LC-11 when grown in the presence of metal analogs of Fe(III)

Metal (1 mM)	Ionic radius (pm)	Generation time (min)		Decrease in growth yield (%)	
		(+) Fe	(−) Fe	(+) Fe	(−) Fe
Fe(III)	64.5	58	90	0	0
Cr(III)	61.5	90	90	78	61
Al(III)	53.5	63	78	0	0
In(III)	80.0	76	—	21	96
Ga(III)	62.0	193	—	91	100
Y(III)	90.0	84	84	55	20
Sc(III)	74.5	110	226	60	50

The ionic radii were obtained from Shannon (1976). Decrease in growth yield was determined by comparison to control cultures which has not received addition of metal analogs of Fe(III). Exponential growth was not observed with In(III); with Ga(III), no growth occurred

iron-limited cells approached the levels observed in the control cultures (Fig. 1 and Table 1). In the absence of added iron, In(III) decreased cell growth markedly and the culture was observed not to attain exponential growth (Fig. 1b). Interestingly, maximum cell yield was not affected by addition of Al(III) to the cultures, either with or without added iron (Table 1). Furthermore, the rate of growth in iron-limited medium containing Al(III) was greater than the control culture without added iron (Table 1).

Of the group III-B elements tested, the addition of Sc(III) to the growth medium showed a much greater increase in growth lag and decrease in the rate of growth in comparison to the medium containing Y(III) (Fig. 1c and Table 1).

Pseudobactin production

We found that pseudobactin was formed only in iron-deficient control cultures or cultures supplemented with either Cr(III), Sc(III) or Y(III). The presence of pseudobactin was confirmed in these cultures by isolating the siderophore by solvent extraction and recording the visible absorption spectrum of ferric pseudobactin (Fig. 2). Ferric pseudobactin formed in iron-deficient medium supplemented with Cr(III), Sc(III), or Y(III) produced virtually identical visible absorption spectra with an absorption maximum at 400 nm. These data agree with other reports on aspects of the chemical characteristics of ferric pseudobactin (Buyer et al. 1990; Teintze et al. 1981). The lack of formation of pseudobactin in all of the other metal-supplemented liquid cultures was substantiated by the apparent lack of fluorescent pigment produced in iron-deficient and iron-sufficient liquid and solid-plating cultures amended with either Al(III), In(III), or Ga(III).

The levels of pseudobactin produced in liquid cultures under iron-deficient conditions in the presence of 1 mM Cr(III), Sc(III) and Y(III) varied considerably (Fig. 3 and Table 2). The highest concentration of pseudobactin was produced after 120 h of incubation by iron-stressed, Cr(III)-supplemented cultures of LC-11, whereas the lowest levels of siderophore were observed to be formed by iron-deficient control and Y(III)-supplemented cells. Under all of these conditions of iron-

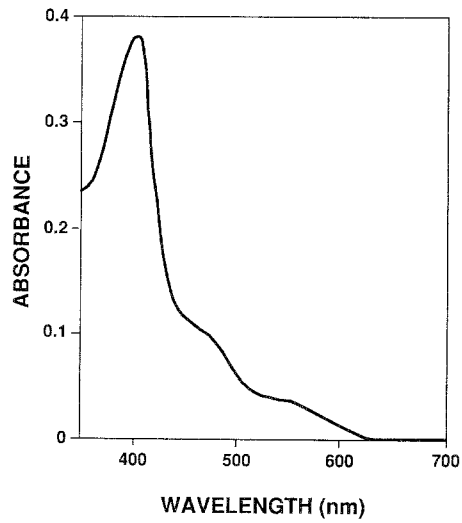

Fig. 2. Visible absorption spectrum of an aqueous solution of ferric-pseudobactin at pH 6.5. The siderophore was isolated from iron-limited cultures of *Pseudomonas* strain LC-11 grown in the presence of 1 mM Sc(III)

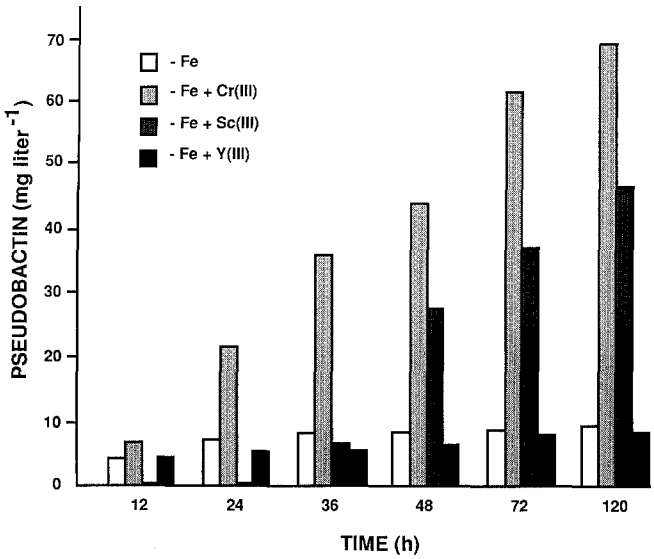

Fig. 3. Production of ferric-pseudobactin by *Pseudomonas* strain LC-11 in iron-limited control cultures and cultures supplemented with 1 mM Cr(III), Sc(III), or Y(III). Mean values of duplicate experiments are plotted

Table 2. Pseudobactin synthesis by *Pseudomonas* LC-11 in low-iron medium containing Cr(III), Sc(III), or Y(III)

Metal added to Fe-limited media	Rate of pseudobactin synthesis ($mg \cdot l^{-1} \cdot h^{-1}$)	Yield of pseudobactin/10^8 cells ($mg \cdot l^{-1}$)
None (control)	0.07	0.15
Cr(III)	0.8	15.9
Sc(III)	0.8	2.9
Y(III)	0.11	0.6

Measurements of the rate of parendobactin synthesis were taken between 8–72 h of culture growth, while measurements of pseudobactin production were taken at 72 h of growth

deficiency and Cr(III) and Y(III) supplementation, pseudobactin formation commenced after approximately 12 h of culture incubation which was at or near the cessation of the logarithmic phase of growth (Figs. 1 and 3). Iron-stressed, Sc(III)-supplemented cultures, which formed the second highest yield of siderophore (Table 2), initiated pseudobactin synthesis after 36 h of incubation. This delayed synthesis parallels the extended lag observed under these culture conditions; however, siderophore production was initiated during maximal cellular activity in the mid-logarithmic phase of growth (Figs. 1 and 3). Once derepression of siderophore synthesis occurred, however, the rate of pseudobactin synthesis in Sc(III)-treated cultures was identical to that in Cr(III)-treated cultures. Pseudobactin yields varied markedly between the two metal-supplemented cultures, i.e. in the presence of Cr(III), there was a five-fold increase in yield in comparison to the yield obtained by cells grown with Sc(III) (Table 2).

Discussion

Chromium compounds have widespread industrial applications, consequently Cr exists in the trivalent or hexavalent form as a contaminant in certain terrestrial and aquatic environments (Papp 1985). The Cr(VI) form is considered to be more toxic than Cr(III) to bacteria (Ross et al. 1981; Ishibashi et al. 1990). Toxicity in microorganisms attributed to Cr(III) is not well understood. The *Pseudomonas* sp. strain LC-II was found to tolerate 5–10 mM Cr(III) in both liquid and solid-plating metal-treated media. It has been proposed that Cr(III) forms large hydroxy polymers at neutral pH levels and would, therefore, be less accessible to cells than the free ion (Ross et al. 1981; Neilands 1984). Under certain conditions, if Cr(III) co-polymerized with Fe(III), this removal of Fe(III) from solution may explain the observed high levels of siderophore formed by strain LC-11 on Cr(III)-supplemented media. This elicitation of siderophores may be at least one mechanism by which the LC-11 isolate could tolerate Cr(III) in the soil.

Fe(III) and Cr(III) exhibit similar solubility characteristics (Neilands 1984). The hexacoordinate ionic radius of Cr(III) is nearly identical to that of high-spin Fe(III) (Table 1). Also, both ions have three positive charges and form octahedral complexes with oxygen, nitrogen, and sulfur ligand matrices (Hughes and Poole 1989). This suggests that Cr(III) may mimic Fe(III) in vivo which would allow complexation of iron binding sites in this pseudomonad isolate. Moreover, because of the kinetic inertness of the Cr(III) complexes (Leong et al. 1974), the exchange of Cr(III) from ligands by Fe(III) would be infinitely slow and could account for an iron-stressed nutritional condition for bacteria. However, we do not believe that the Cr(III)-pseudobactin complex was formed in culture since pseudobactin-producing cultures were fluorescent. Furthermore, culture supernatant fluids from Cr(III)-containing media reacted readily with $FeCl_3$ to form the characteristic brown-red ferric pseudobactin complex. The ferrated form of the complex was a prerequisite for measurement of pseudobactin by absorbance at 400 nm and complex formation was accompanied by the corresponding loss of fluorescence. This observation concerning the apparent lack of Cr(III)-siderophore formation is supported by Meyer and Abdallah (1978) who also found that the addition of Al(III) and Cr(III) to aqueous supernatants containing pseudobactin (pyoverdin) produced no characteristic changes associated with complex formation.

Sc(III), and to a lesser extent Y(III), were found to have a stimulatory effect on pseudobactin synthesis and yield (Fig. 3 and Table 2). Sc(III) and Y(III) are incapable of being reduced because no divalent oxidation state of these metals is known. Because it is the divalent form of iron that functions as co-repressor in the negative regulation of siderophore synthesis (Neilands et al. 1987), trivalent Sc and Y would probably not bind to the repressor protein. Sc(III) and Y(III) possess considerably larger radii than Fe(III) (Table 1) and this may also prohibit effective complexation with repressor.

Cr(III), which was found to elicit hyperproduction of pseudobactin synthesis (Fig. 3 and Table 2), is known to exist in a divalent form. However, because Cr(III) is not known to be reduced to Cr(II) in biological systems, Cr(III) would similarly be unable to function as a co-repressor of siderophore synthesis. These observations offer a mechanism which may account for the marked derepression of pseudobactin synthesis when the LC-11 isolate was grown in the presence of Sc(III), Y(III), and Cr(III).

Our observed antibacterial effects of Sc(III) on the pseudomonad isolate (Fig. 1 and Table 1) contrasts with the results of Rogers (1987) who observed that Sc(III), as a free ion, lacks antibacterial activity in pathogenic serotypes of *E. coli*. However, when Sc(III) is supplied as the Sc(III)-enterobactin complex, *E. coli* is immediately killed. In another study using a strain of *Pseudomonas aeruginosa*, Rogers et al. (1984) reported that the inhibitory effects of Sc(III)-enterobactin on growth are reversed upon addition of deferri-pseudobactin, the natural siderophore formed by that bacterium. We have no evidence that Sc(III)-pseudobactin was produced and we did not investigate the effects of the addition of the Sc(III)-siderophore complex to LC-11 cultures.

The group III-A metals, Al(III), In(III), and Ga(III), were found to repress pseudobactin synthesis totally (Fig. 3). Ga(III), with an ionic radius of 62 pm and possessing a similar preference for oxygen/nitrogen/sulfur ligand matrices (Emery 1986; Hughes and Poole 1989), would be expected to be the metal that most closely mimics Fe(III). Interestingly, Ga(III) at 1 mM concentration was the metal most active in inhibiting the growth of both iron-deficient and iron-sufficient cultures of *Pseudomonas* LC-11 (Fig. 1 and Table 1). Hubbard et al. (1986) found that 9 μM Ga(III) nitrate added to iron-limited, but not iron-replete cultures, decreased growth yields of *E. coli*. While Hubbard et al. (1986) reported that Ga(III) stimulated Fe(III) uptake and was transported by iron-sufficient *E. coli*, Moody and Dailey (1985) found that Ga(III) citrate inhibited Fe(III) uptake and Ga(III) was not transported by *R. sphaeroides*. According to Hubbard and co-workers (1986), their data suggest that Ga(III) affects the low-affinity pathway for iron uptake. The role of Ga(III) in the apparent repression of pseudobactin synthesis in this pseudomonad isolate will remain unresolved until radioactive metal transport assays are performed.

In(III) was observed to have pronounced bacteriostatic activity only when iron was omitted from the culture medium (Fig. 1). Conversely, iron-replete cells in In(III)-treated cultures showed only a slight diminution of growth rate and no increase in the lag phase in comparison to untreated control cultures (Fig. 1 and Table 1). The fact that the inhibitory effect of In(III) on growth of LC-11 was reversed in the presence of iron suggests that its toxicity is related, in part, to its ability to mimic Fe(III) in vivo. The same is true, albeit to a lesser extent, concerning the observed inhibitory effects of Ga(III) and Sc(III) and their reversal of growth inhibition by iron addition (Fig. 1). In(III)-enterobactin complexes have also been reported to have significant bacteriostatic effects in a number of Gram-negative bacterial systems; similarly, the inhibitory effect of the In(III)-siderophore complex on bacterial growth is reversed by the corresponding Fe(III) complex (Rogers 1987).

Al(III), like Cr(III), has been used as an additive to culture media to enhance the production of siderophores (Byers et al. 1967; Neilands 1984); Al(III), even at low growth medium concentration, has been shown to be extremely toxic to a multitude of microbial systems (Bradley and Parker 1968; Johnson and Wood 1990; Pettersson et al. 1985). In contrast, we found that Al(III) was neither growth inhibitory nor stimulatory in pseudobactin synthesis in LC-11 cultures (Figs. 1, 3 and Table 1). Because Al becomes more soluble as acidity increases and is considered to be a major toxic metal in acidic soils and aquatic systems (Cronan et al. 1986; Thomas and Hargrove 1984), we repeated the same experiment with cultures of *Pseudomonas* LC-11 supplemented with 1 mM Al(III) except that the pH was adjusted to 5.5. The response to Al(III) in terms of both growth and siderophore synthesis was the same as that observed for this organism at pH 7.0 (data not shown). Upon examination, it is apparent that Al(III) possesses a smaller ionic radius than Fe(III) (Table 1) and is partitioned in ligand matrices composed of oxygen exclusively, while Fe(III) has the capability of binding to oxygen, nitrogen and sulfur ligands (Hughes and Poole 1989; Martin 1986). The specific chemical characteristics of Al(III) which limit biomimetic activity for Fe(III) in this *Pseudomonas* sp. is unexplained at this time.

To our knowledge, this is the first report to describe the physiological response of a microorganism, in terms of growth and siderophore formation, when grown in the presence of metals that mimic Fe(III). Although the metals tested have the same oxidation states, relatively similar ionic radii, and some form octahedral complexes by binding to the same ligand groups, markedly different effects on bacteriostasis and siderophore formation were demonstrated. Through further research on the activities of Fe(III) analogs on both high- and low-affinity iron acquisition systems, our understanding of iron physiology will be greatly enhanced.

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