Iron-regulated salicylate synthesis by *Pseudomonas* spp.

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Two iron-regulated compounds have been found in acidified ethyl acetate extracts from culture supernatants of *Pseudomonas aeruginosa* and *Pseudomonas cepacia* type-strains. Synthesis of both compounds paralleled iron-deficient growth, and was repressed in the presence of 100 μM-FeCl₃. Yields of these substances varied among different strains and attained maximum levels during stationary phase. Thin layer chromatographic analysis in five different solvent systems revealed that the slower-moving compound chromatographed as two distinct bands, and showed Rₚ values and spectral properties similar to pyochelin. The faster-moving compound co-migrated as a single band with a standard of commercial salicylic acid in each of the chromatographic systems tested. Moreover, a molecule with an identical Rₚ was also produced by *Pseudomonas fluorescens* CHA401, which is known to synthesize salicylic acid as the only siderophore during iron-limited growth. Spectrophotometric and spectrofluorometric titrations led to the identification of this iron-regulated compound as salicylic acid, in agreement with the structure deduced from ¹H-NMR and mass spectroscopy. The identity of the *P. cepacia* siderophore azurechelin as salicylic acid was also conclusively demonstrated. Salicylic acid, like pyochelin and pyoverdin, promoted *P. aeruginosa* growth in an iron-depleted medium. These results are consistent with a putative siderophore activity for salicylic acid, i.e. azurechelin, as has been demonstrated for *P. aeruginosa, P. fluorescens* and *P. cepacia*. Thus, salicylic acid is likely to act as a siderophore in more than one species belonging to the genus *Pseudomonas*.

Introduction

In spite of its natural abundance, iron [Fe(III)] is poorly available for most living organisms (Cox, 1989). To counter Fe(III) deficiency almost all bacteria and fungi have evolved high-affinity Fe(III) transport systems based on the synthesis of low-molecular-mass chelators (siderophores) and of their cognate receptors (Neilands, 1981, 1982). Siderophore-mediated Fe(III) acquisition is essential not only for the survival of micro-organisms in the environment, but also for the ability of bacterial and fungal parasites to establish and maintain infections (Cox, 1989; Weinberg, 1978).

Although the genus *Pseudomonas* includes several taxonomically and ecologically distinct bacteria, some species belonging to the first and second homology groups (Palleroni, 1984), i.e. *P. aeruginosa* and *P. cepacia*, synthesize a common siderophore, termed pyochelin, consisting of a salicyl-substituted cysteinyl peptide (Cox & Graham, 1979; Cox,* et al., 1981; Sokol. 1986). Apart from pyochelin, fluorescent pseudomonads synthesize additional siderophores generally termed pyoverdins (or pseudobactins in plant-related isolates) (Abdallah, 1991), whereas *P. cepacia* strains may also produce cepabactin (Meyer *et al.*, 1989) and azurechelin (Sokol *et al.*, 1992). Pyochelin has a relatively low affinity for Fe(III) (Kₛ = 2 × 10⁵) which is bound in a 2:1 stoichiometric ratio (Cox & Graham, 1979; Visca *et al.*, 1992b). Ferripyochelin uptake occurs via two distinct outer-membrane receptor proteins (Heinrichs *et al.*, 1991; Sokol & Woods, 1983). Pyochelin has been shown to form functionally active complexes with a variety of transition metals (Visca *et al.*, 1992b), and has been associated with the virulence of *P. aeruginosa* in experimental mouse infections (Cox, 1982) and of *P. cepacia* in respiratory tract infections (Sokol & Woods, 1988). Furthermore, a siderophore-like activity for salicylic acid, the biosynthetic precursor of pyochelin (Ankenbauer, & Cox, 1988), has recently been recognized in *P. fluorescens* (Meyer *et al.*, 1992).

In this paper we show that salicylic acid is not only an intermediate in pyochelin synthesis, but represents an authentic, endogenous siderophore for some *P. aeruginosa, P. fluorescens* and *P. cepacia* isolates. We also
demonstrate that azurechelin, a siderophore of formerly unknown structure produced by P. cepacia (Sokol et al., 1992), is in fact salicylic acid.

Methods

Bacterial strains. P. aeruginosa PA01 (ATCC 15692), which produces pyoverdin (Abdallah, 1991) and pyochelin (Cox & Graham, 1979), was obtained from the American Type Culture Collection. P. aeruginosa PA46U was isolated in the Institute of Microbiology of the University of Rome "La Sapienza" from the urine of an infected patient; this isolate has been shown to synthesize both pyochelin and pyoverdin (Visca et al., 1991). P. fluorescens CHAO, a soi isolate producing both pyoverdin and salicylate (Stutz et al., 1986; Meyer et al., 1992), and the pyoverdin-defective mutant CHAA01 (Keel et al., 1989) derived from this strain by Tn5 insertion mutagenesis were kindly provided by D. Haas, Mikrobiologisches Institut, ETH, Zürich, Switzerland. The P. cepacia strains Pc275c and Pc710m, producing both pyochelin and azurechelin, and K30-6 and H1724-1, producing azurechelin only, were a gift of P. Sokol, Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, Alberta, Canada (Sokol et al., 1992).

Media and culture conditions. All pseudomonads, except P. fluorescens, were routinely grown in DCAA, containing 0.5% (w/v) Casamino acids (Difco) in deionized, double distilled water, made metal-free by treatment with Chelex 100 resin (Bio-Rad). The activated resin was added to the Casamino acids solution (5 g resin per litre medium) and the pH was adjusted to 7.2 by the addition of a few drops of 1 M-NaOH. After 16 h moderate stirring at 4°C the resin was removed by filtration through Whatman no. 1 filter paper and the medium was sterilized by autoclaving. The medium was supplemented aseptically with 0.4 mM-MgCl2 immediately prior to inoculation. After Chelex 100 treatment, the Fe(II1) content of the acidified supernatants obtained after centrifugation (5000 g) was determined by stepwise titrations with Fe(II1) specific reactivity of N-methylthiazolidine rings yielding black spots when sprayed with ammoniacal silver nitrate reagent (Kirchner, 1967; Ankenbauer & Cox, 1988); (iv) chromatographic mobility (Rf = 0.35-0.40 in solvent I (Ankenbauer et al., 1991)). Salicylic acid from TLC plates was qualitatively characterized by the blue fluorescence emission under UV light (Feigl, 1960); (ii) iron-binding capacity resulting in red-violet spots when sprayed with 0.1 M-FeCl3 in 0.1 M-HCl (Cox & Graham, 1979); (iii) chromatographic mobility in comparison with a standard of commercial salicylic acid (Rf = 0.60).

For TLC analysis five different solvent systems were used: solvent I, chloroform/acetic acid/ethanol (90:5:2.5, by vol.); solvent II, chloroform/acetic acid (90:1, v/v); solvent III, chloroform/ethanol (4:1, v/v); solvent IV, 2-propanol/25% (w/v) ammonium hydroxide/ water (100:10:10, by vol.); solvent V, benzene/tholuene/acetic acid (2:2:1, by vol.). Solvents I-IV were from Cox & Graham (1979) and Meyer et al. (1989), while solvent V was from Saxena et al. (1986).

Quantitative assays of pyochelin and salicylic acid were done by scraping the fluorescent spots from TLC plates loaded with extracts from 50 ml cultures. The compounds were eluted from the silica gel by extracting twice with methanol. The methanol extracts were dried under vacuum and the amounts of pyochelin and salicylic acid determined by spectrophotometric titrations of the desferri-iron-saturated forms in the 230-630 nm wavelength range, in comparison with known standards.

Large-scale preparations of pyochelin and salicylic acid were made by growing strains in stirred 1 litre flasks containing 250 ml DCAA or SM9CAA; the supernatants obtained after centrifugation were acidified, extracted with ethyl acetate, and sequentially chromatographed in solvent I and V as described above. Preparative HPLC was performed according to minor modifications of the procedures of Meyer et al. (1992). When possible, salicylate and pyochelin were protected from light and stored in dark containers.

Chemical analysis. Absorption spectra in the visible and UV ranges (230-630 nm) were measured at 20°C with a Beckman model 25 spectrophotometer. Titrations were performed at pH 6 by stepwise addition of FeCl3 from solutions prepared in equimolar concentrations of HCl. Under the solvent condition chosen, the solubility of pyochelin, salicylate and their complex with Fe(III) is high and the absorption spectra of desferri-forms and complexes with Fe(III) have been reported (Cox & Graham, 1979; Meyer et al., 1992; Ungar et al., 1952; Visca et al., 1992b).

Fluorescence excitation and emission spectra were performed in 97% (v/v) methanol, 30 mM-Tris (pH 11) using a Jasco FP-770 spectrofluorometer. High resolution electron impact (HREI) mass spectra were obtained on an Apex 47e Spectrospin Bruker ion cyclotron resonance spectrometer.

1H-NMR analyses of compounds dissolved in CD3OD were performed in a Bruker 500 spectrometer using TMS as internal standard. The identification of salicylic acid was made by direct comparison with the commercial standard.

Measurement of bacterial growth rates. P. aeruginosa strains from SM9 agar plates were inoculated into 25 ml DCAA and grown overnight at 37°C with vigorous shaking to deplete cells of essential metals. Bacterial cells were centrifuged (20 min, 2500 g, 37°C in a Heraeus Omnimulf 2.0 RS centrifuge), resuspended in pre-warmed DCAA to an OD660 of 1.0, and 0.1 ml aliquots of bacterial suspension were used to inoculate 25 ml pre-warmed DCAA in 350 ml Erlenmeyer
Salicylate synthesis by pseudomonads

Figure 1. Thin layer chromatograms of acidified ethyl acetate extracts from cultures of different Pseudomonas strains grown in Fe(III)-deficient and Fe(III)-rich medium. In the Fe(III) rich conditions FeCl₃ was added to DCAA or SM9CAA at a final concentration of 100 µM (see Methods). Lanes: 1 and 2, P. aeruginosa PA01 in Fe(III)-poor and Fe(III)-rich medium, respectively; 3 and 4, P. aeruginosa PA46U in Fe(III)-poor and Fe(III)-rich medium, respectively; 5 and 6, P. fluorescens CHA401 in Fe(III)-poor and Fe(III)-rich medium, respectively; 7 and 8, P. cepacia Pc275c in Fe(III)-poor and Fe(III)-rich medium, respectively; 9 and 10, P. aeruginosa PA01 in Fe(III)-poor and Fe(III)-rich medium, respectively; 11-14, 5, 10, 20 and 40 nmol commercial salicylic acid, respectively. (a) Chromatograms visualized by exposure to UV light; (b) chromatograms developed by spraying with 0.1 M FeCl₃ in 0.1 M HCL; (c) chromatograms developed by spraying with the ammoniacal silver nitrate reagent for thiazolidine groups (Kirchner, 1967). Abbreviations: F, solvent front; O, origin of migration; A₁, A₂ and B, compounds A₁, A₂ and B. Lanes 1 to 10 were loaded with the equivalent of 3.5 ml culture extracts.

Results and Discussion

Fe(III)-regulated compounds in ethyl acetate extracts of supernatants from Pseudomonas spp.

TLC analysis in solvent I of supernatants from cultures of different Pseudomonas strains grown in Fe(III)-depleted and Fe(III)-rich media is shown in Fig. 1. Three different Fe(III)-regulated compounds were observed, provisionally termed A₁, A₂ and B, with Rₚ values of 0.35, 0.39 and 0.60, respectively. Compounds A₁ and A₂ exhibited green-grey fluorescence under UV light, turned brown-red on reaction with Fe(III), and gave a positive reaction for thiazolidine rings which are known to form black silver mercaptide salts during alkaline hydrolysis in the presence of Ag(I) (Kirchner, 1967; Ankenbauer et al., 1988). Compound B exhibited chromatographic mobility, fluorescence emissions and chemical reactivity identical to that of commercial salicylic acid (Fig. 1) and resembled that described by Sokol et al. (1992) for azurechelin. Emission of blue-violet fluorescence under UV light is a typical spot test in the qualitative analysis of salicylates.

flasks. Cultures were incubated at 37 °C with aeration in an orbital shaker (New Brunswick, model G25) at 250 rev. min⁻¹ and 1 ml aliquots were removed at hourly intervals for measurement of OD₆₂₀. The rate of biomass increase was estimated over 6 h after inoculation and the mean generation time was determined as previously described (Visca et al., 1992b).

pyoverdin-defective derivative CHA401. However, the level of compounds A₁ and A₂ was significantly lower in P. cepacia than in P. aeruginosa strains. Compound B exhibited an evident blue-violet fluorescence under UV light and reacted with Fe(III) yielding red-violet spots, but did not turn black upon reaction with the ammoniacal silver nitrate reagent for thiazolidine groups. Compound B was synthesized by each of the species tested. Remarkably, synthesis of compounds A₁, A₂ and B appeared to be repressed in cultures supplemented with 100 µM-FeCl₃. Compounds A₁ and A₂ displayed the same chromatographic mobility and chemical reactivity as pyochelin from P. aeruginosa PA01 which, under the solvent conditions chosen, is known to migrate as two distinct Fe(III) and Ag(I) reactive forms of Rₚ 0-30-0-40, probably corresponding to pyochelin I (Rₚ 0-35) and pyochelin II (Rₚ 0-40) (Ankenbauer et al., 1988). Compound B exhibited chromatographic mobility, fluorescence emissions and chemical reactivity identical to that of commercial salicylic acid (Fig. 1) and resembled that described by Sokol et al. (1992) for azurechelin. Emission of blue-violet fluorescence under UV light is a typical spot test in the qualitative analysis of salicylates.
Table 1. TLC R_f values of Fe(III)-regulated compounds in ethyl acetate extracts of culture supernatants from different Pseudomonas spp.

<table>
<thead>
<tr>
<th>Compound</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0.60</td>
<td>0.69</td>
<td>0.57</td>
<td>0.64</td>
<td>0.61</td>
</tr>
<tr>
<td>A_1</td>
<td>0.39</td>
<td>0.33</td>
<td>0.37</td>
<td>0.55</td>
<td>0.32</td>
</tr>
<tr>
<td>A_2</td>
<td>0.35</td>
<td>0.25</td>
<td>0.35</td>
<td>0.32</td>
<td>0.21</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>0.60</td>
<td>0.69</td>
<td>0.56</td>
<td>0.64</td>
<td>0.61</td>
</tr>
</tbody>
</table>

*See Methods for the composition of solvents and TLC plates used.

(Feigl, 1960). The hypothesis that compound B and azurechelin might correspond to the same molecule, i.e. salicylic acid, was strengthened by the observation that compound B was the only Fe(III)-regulated substance in culture extracts of *P. fluorescens* CHA401, which is known to synthesize salicylic acid as the only siderophore during Fe(III)-limited growth (Meyer et al., 1992). As in the case of azurechelin (Sokol et al., 1992), compound B gave a negative reaction in the Arnow (1937) assay for catechols and in the Gillan et al. (1981) assay for hydroxamates, as expected for salicylic acid.

Development of TLC plates in five different solvent systems consistently resolved the three Fe(III)-regulated bands as detectable by fluorescence emission and reactivity with Fe(III) and/or Ag(I) ions (Table 1). Compounds A_1 and A_2 migrated in all solvents as two discrete bands, with R_f values roughly corresponding to those reported for pyochelin (Cox & Graham, 1979; Meyer et al., 1989). The chromatograms also confirmed the virtual identity of compound B as salicylic acid, both migrating with the same R_f in all solvent systems tested.

The synthesis of both compounds in Fe(III)-depleted media paralleled bacterial growth and attained the maximum during the stationary phase at which stage the level of compounds A_1–A_2 (pyochelin) and B (salicylic acid) varied in a range 0–26 μM and 27–59 μM, respectively, depending upon the strain (data not shown).

TLC analysis of culture extracts from 121 clinical isolates of *P. aeruginosa* revealed that only 13.2% produced detectable amounts of compound B, while 97.5% produced compounds A_1 and A_2. It is interesting to note that one out of the three *P. aeruginosa* isolates defective in the synthesis of compounds A_1 and A_2 was found to release very large amounts of compound B.

Chemical characterization of Fe(III)-regulated compounds in ethyl acetate extracts from Pseudomonas spp.

The results presented above strongly support the hypothesis that compound B and azurechelin may correspond to salicylic acid and prompted us to perform spectrophotometric analyses of these molecules and their complexes with Fe(III). The UV-visible spectra (230–630 nm) were obtained in a methanol solution at pH 6.0 containing compound B adjusted to a concentration spectrophotometrically equivalent to 100 μM-salicylic acid (Fig. 2). Under these conditions compound B displayed an absorption spectrum identical to salicylic acid with maxima at 298 nm (ε = 5220 M⁻¹ cm⁻¹) and
240 nm ($\varepsilon = 8440 \text{ M}^{-1} \text{ cm}^{-1}$) as shown by spectra B and S in Fig. 2. Saturation of compound B and salicylic acid with an equimolar amount of Fe(III) caused an evident spectral transition for both compounds with the presence of an additional absorption maximum in the visible region at approximately 550 nm. To analyse the formation of complexes between salicylic acid and Fe(III) titration experiments were performed (spectra S,Fe* and SFe* in Fig. 2b). The spectral changes observed at increasing salicylate/Fe(III) molar ratios suggest the formation of complexes with different stoichiometry, namely 1:1, 2:1 and 3:1, characterized by absorption maxima in the visible region at 555 nm ($\varepsilon = 1600 \text{ M}^{-1} \text{ cm}^{-1}$), 516 nm ($\varepsilon = 837 \text{ M}^{-1} \text{ cm}^{-1}$) and 490 nm ($\varepsilon = 710 \text{ M}^{-1} \text{ cm}^{-1}$), respectively. It should also be pointed out that salicylate is known to provide a very efficient Fe(III)-binding centre and generate salicylate–Fe(III) complexes characterized by high stability constants, their log $K_p$ ($1 \text{ M}, 25 \text{ °C}$) ranging from 158 for the monosalicylate–Fe(III) complex to 353 for the trisalicylate–Fe(III) complex (Martell & Smith, 1977).

The UV-visible absorption spectra of compounds A1 and A2 were determined under the same solvent conditions described above. The results obtained (spectrum A in Fig. 2a) indicate that compounds A1 and A2 correspond to pyochelin, since each showed typical absorption maxima at 250 and 310 nm, as previously reported by Cox & Graham (1979). Moreover, compounds A1 and A2 did not exhibit any difference in their spectroscopic properties in the UV-visible range (data not shown), as reported by Ankenbauer et al. (1988) for the two forms of pyochelin.

Our assumption that compound B and azurechelin are both salicylic acid was further strengthened by fluorometric analyses (Fig. 3). The analyses were carried out by dissolving both compounds in 97% (v/v) methanol, 30 mM-Tris, pH 11.0, to achieve a final concentration of 1.66 μM. The results obtained show that compound B (from P. cepacia Pc275c) displayed excitation and emission spectra identical to those observed for salicylic acid, with excitation and emission maxima at 295 and 404 nm, respectively.

The UV-visible and fluorescence spectra of compounds A1, A2, and B were compared for all the strains examined. Each compound appeared to be homogeneous as no difference was observed among compounds A1, A2 and B extracted from the different Pseudomonas strains. The possibility that compound B (salicylic acid) could originate from spontaneous degradation of compounds A1 and A2 (pyochelin) was ruled out by our observations that prolonged incubation of the last two substances in acid (pH 1-5) or alkali (pH 12-8) did not generate compound B, as detectable after acidified ethyl acetate extraction and TLC analysis.

That compound B and azurechelin are both salicylic acid was conclusively confirmed by means of 1H-NMR and HREI mass spectroscopy. The HREI spectroscopic analysis of freshly purified compound B resembled that of commercial salicylic acid, both compounds showing the same fragmentation pattern with main ions at m/z values of 138, 94 and 66. The 1H-NMR spectral characteristics of compound B dissolved in CD3OD are given in Fig. 4, together with the deduced formula. The HREI and NMR data together with those deduced from TLC analysis and UV-visible and fluorescence spectroscopy, led us to the unequivocal conclusion that compound B and azurechelin are salicylic acid.

**Growth promoting activity of salicylic acid and its complexes with Fe(III)**

The ability of salicylic acid to function as a siderophore has already been shown in P. fluorescens (Meyer et al., 1992) and P. cepacia (Sokol et al., 1992). To investigate the involvement of salicylic acid in Fe(III) transport by P. aeruginosa, we compared the growth rates of strains PA01 and PA46U in Fe(III)-depleted medium (DCAA) supplemented with salicylate and the salicylate/Fe(III) 3:1 complex. The results obtained (Table 2) indicate that
the functional effects of salicylate in \textit{Pseudomonas} spp. and other bacterial genera is worth noting. Salicylic acid is a precursor of pyochelin in \textit{P. aeruginosa} (Ankenbauer & Cox, 1988) and mycobactin in \textit{Mycobacterium} spp. (for a review see Ratledge, 1987) and is synthesized by pseudomonads and mycobacteria in response to Fe(III)-limited growth (Meyer et al., 1992; Ratledge & Winder, 1962). Moreover, salicylate is endowed with a siderophore-like activity in \textit{Mycobacterium} spp. (Ratledge, 1987; Ratledge & Winder, 1962; Ratledge et al., 1974), \textit{Azospirillum lipoferum} (Saxena et al., 1986), \textit{P. aeruginosa} (Meyer, 1992) and \textit{P. fluorescens} (Meyer et al., 1992), although its Fe(III)-sequestering properties in the presence of competing or precipitating ligands are questionable (Ratledge et al., 1974). The mechanism of translocation of the Fe(III)–salicylate complex(es) into the cell is unclear. It is tempting to speculate that they enter the cell via specific receptors. Due to the structural similarities of salicylate with pyochelin, obvious candidates for Fe(III)–salicylate transport, at least in \textit{P. aeruginosa} PAO1, are the 14 kDa and 75 kDa ferripyochelin uptake proteins (Heinrichs et al., 1991; Sokol & Woods, 1983). However, similarly to pyochelin, salicylate and its complexes with Fe(III) are poorly water-soluble (Ratledge et al., 1974) and might therefore associate with the lipid layer of the bacterial outer membrane. It will be of interest to determine whether salicylate may induce the expression of specific iron-regulated outer-membrane protein(s) involved in ferrisiderophore uptake, as already shown for pyochelin and pyoverdin in \textit{P. aeruginosa} (Gensberg et al., 1992).

Taken together, the results presented here demonstrate that \textit{P. aeruginosa} and \textit{P. cepacia}, as well as \textit{P. fluorescens}, synthesize salicylate during Fe(III)-limited growth. Indeed, UV-visible spectroscopic properties determined for compound B are in line with those previously published for salicylic acid (Boltz & Schenk, 1963; Ungar et al., 1952), as are data derived from fluorescence, HREI and NMR analysis (Weissler & White, 1963; Marshall & Ratledge, 1972). We have also demonstrated that azurechelin, formerly identified in \textit{P. cepacia} by Sokol et al. (1992) is in fact salicylic acid. In all the strains examined production of salicylate occurred in response to Fe(III)-starvation, thus leading to the hypothesis that it is involved in Fe(III)-uptake. This contention was supported by the significant growth-promoting activity of salicylate and its Fe(III)-complex under conditions of Fe(III)-deficiency.

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Table 2. Effect of salicylate and its Fe(III)-complex on \textit{Pseudomonas aeruginosa} growth in metal-poor medium (DCAA)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nil</th>
<th>Fe(III)</th>
<th>Salicylate</th>
<th>Fe(III)–salicylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA01</td>
<td>75.2 (± 3.0)</td>
<td>48.1 (± 0.9)</td>
<td>68.4 (± 2.4)</td>
<td>42.3 (± 1.7)</td>
</tr>
<tr>
<td>PA46U</td>
<td>68.7 (± 4.5)</td>
<td>43.6 (± 2.3)</td>
<td>61.0 (± 3.9)</td>
<td>39.8 (± 1.1)</td>
</tr>
</tbody>
</table>

Fig. 4. $^1$H-NMR spectrum (a) and deduced formula (b) of compound B.

growth of both \textit{P. aeruginosa} strains under conditions of limiting Fe(III) is promoted by salicylate and, to a greater extent, by its complex with Fe(III). Since both strains were unable to use salicylate as their sole carbon source, such growth stimulation might be ascribed to salicylate-mediated Fe(III) acquisition. These functional effects of salicylate and its Fe(III)-complexes parallel those previously demonstrated for pyoverdin and pyochelin and their ferri-forms (Visca et al., 1992b).

The interesting parallels observed for the synthesis and
References


