Iron-regulated salicylate synthesis by *Pseudomonas* spp.

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(Received 23 December 1992; revised 3 April 1993; accepted 22 April 1993)

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 R_f 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 R_f 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 pyoverdine, 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) ($K_f = 2 \times 10^5$) 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

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Abbreviation: HREI, high resolution electron impact.

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* PAOI (ATCC 15692), which produces pyoverdine (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 pyoverdine (Visca *et al.*, 1991, 1992a). *P. fluorescens* CHAO, a soil isolate producing both pyoverdine and salicylate (Stutz *et al.*, 1986; Meyer *et al.*, 1992), and the pyoverdine-defective mutant CHA401 (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(III) content of DCAA, determined by means of an atomic absorption spectrophotometer (Perkin-Elmer model 360), was < 0.1 µM. Alternatively, M9 minimal medium (Guterman, 1973) was used after supplementation with 20 mM-sodium succinate as carbon source (SM9). The M9 basal solution was also treated with 5 g Chelex 100 l⁻¹ as described above. When required, media were solidified by the addition of 10 g agar l⁻¹ (N. 1; Oxoid). For *P. fluorescens* isolates, which grew poorly in DCAA, the Fe(III)-depleted M9 medium supplemented with 20 mM-sodium succinate and 5 g Casamino acids l⁻¹ (SM9CAA) was used.

To deplete the cells of intracellular Fe(III) pools, bacteria were first cultured on SM9 agar plates, suspended in a small volume of sterile, double distilled water to an OD₆₂₀ of 0.5 and inoculated in DCAA or SM9CAA (approximately 10⁶ cells ml⁻¹). The culture was incubated at 37 °C in a rotary shaker at 250 rev. min⁻¹ until late exponential phase. This culture was subsequently used as inoculum to achieve a final concentration of approximately 10⁶ cells ml⁻¹.

All glassware was rendered iron-free by 24 h treatment with 0.1 M-HCl, rinsed once in distilled water and finally rinsed in deionized, double distilled water before sterilization. All media and solutions were prepared with deionized, double distilled water. Commercial salicylic acid and its sodium salt were purchased from Aldrich.

Extraction and characterization of pyochelin and salicylic acid. Pyochelin and salicylic acid were isolated by ethyl acetate extraction of acidified supernatants obtained after centrifugation (5000 g, 30 min, 4 °C) of 36 h bacterial cultures in DCAA or SM9CAA. Briefly, the spent medium was adjusted to pH 1.5–2.0 with 1.0 M-HCl and extracted twice with 0.4 vols ethyl acetate. After evaporation of the organic phase under vacuum, the dry residue was resuspended in a small volume of methanol and applied in triplicate to a Silica gel G thin layer

chromatography (TLC) plate which was developed in solvent I (chloroform/acetic acid/ethanol, 90:5:2.5, by vol.).

Pyochelin from TLC plates was qualitatively characterized by: (i) yellow-green fluorescence emission under UV light (Cox & Graham, 1979); (ii) iron binding capacity resulting in red-brown spots when sprayed with 0.1 M-FeCl₃ in 0.1 M-HCl (Cox & Graham, 1979); (iii) specific reactivity of *N*-methylthiazolidine rings yielding black spots when sprayed with ammoniacal silver nitrate reagent (Kirchner, 1967; Ankenbauer & Cox, 1988); (iv) chromatographic mobility [*R_F* = 0.35–0.40 in solvent I (Ankenbauer *et al.*, 1991)]. Salicylic acid from TLC plates was qualitatively characterized by (i) 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 (Meyer *et al.*, 1992); (iii) chromatographic mobility in comparison with a standard of commercial salicylic acid (*R_F* = 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/toluene/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- and 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 solvents 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 complexes 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 Omnifuge 2.0 RS centrifuge), resuspended in pre-warmed DCAA to an OD₆₂₀ of 1.0, and 0.1 ml aliquots of bacterial suspension were used to inoculate 25 ml pre-warmed DCAA in 350 ml Erlenmeyer

Fig. 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 FeCl_3 was added to DCAA or SM9CAA at a final concentration of $100 \mu\text{M}$ (see Methods). Lanes: 1 and 2, *P. aeruginosa* PAO1 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. cepacia* H1724-1 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- FeCl_3 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; A_1 , A_2 and B, compounds A_1 , A_2 and B. Lanes 1 to 10 were loaded with the equivalent of 3.5 ml culture extracts.

flasks. Cultures were incubated at 37°C with aeration in an orbital shaker (New Brunswick, model G25) at $250 \text{ rev. min}^{-1}$ and 1 ml aliquots were removed at hourly intervals for measurement of OD_{620} . 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).

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 A_1 , A_2 and B, with R_F values of 0.35, 0.39 and 0.60, respectively. Compounds A_1 and A_2 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). Compounds A_1 and A_2 were produced by all the strains examined, except *P. fluorescens* CHAO and its

pyoverdine-defective derivative CHA401. However, the level of compounds A_1 and A_2 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 A_1 , A_2 and B appeared to be repressed in cultures supplemented with $100 \mu\text{M}$ - FeCl_3 . Compounds A_1 and A_2 displayed the same chromatographic mobility and chemical reactivity as pyochelin from *P. aeruginosa* PAO1 which, under the solvent conditions chosen, is known to migrate as two distinct Fe(III) and Ag(I) reactive forms of R_F 0.30–0.40, probably corresponding to pyochelin I (R_F 0.35) and pyochelin II (R_F 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.

Compound	R_F value in solvent*				
	I	II	III	IV	V
B	0.60	0.69	0.57	0.64	0.61
A ₁	0.39	0.33	0.37	0.55	0.32
A ₂	0.35	0.25	0.35	0.32	0.21
Salicylic acid	0.60	0.69	0.56	0.64	0.61

* 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 A₁ and A₂ 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 A₁–A₂ (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 A₁ and A₂. It is interesting to note that one out of the three *P. aeruginosa* isolates defective in the synthesis of compounds A₁ and A₂ 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 ($\epsilon = 5220 \text{ M}^{-1} \text{ cm}^{-1}$) and

Fig. 2. (a) Absorption spectra of methanolic solutions at pH 6 of (A) compound A from *P. aeruginosa* PA46U, (B) compound B from *P. cepacia* PC275c, and (BFe) compound B saturated with a theoretically equimolar amount of FeCl_3 . The concentration of compound B was spectrophotometrically adjusted to a value approximately equivalent to 100 μM -salicylic acid. (b) Titration of a methanolic solution of commercial salicylic acid with FeCl_3 . S, 100 μM -salicylic acid; S_3Fe , S_2Fe and SFe , 100 μM -salicylate-Fe(III) complexes in 3:1, 2:1 and 1:1 stoichiometric ratios, respectively; S_3Fe^* , S_2Fe^* and SFe^* , 800 μM -salicylate-Fe(III) complexes in 3:1, 2:1 and 1:1 stoichiometric ratios, respectively.

Fig. 3. Fluorescence spectra of 1.66 μM commercial salicylic acid (*a, b*) and compound B from *P. cepacia* Pc275c (*c, d*) dissolved in 97% methanol, 30 mM-Tris, pH 11. (*a, c*) Excitation spectra with emission measured at 405 nm; (*b, d*) emission spectra upon excitation at 295 nm.

240 nm ($\epsilon = 8440 \text{ M}^{-1} \text{ cm}^{-1}$) 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_3Fe^* , S_2Fe^* and SFe^* in Fig. 2*b*). 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 ($\epsilon = 1600 \text{ M}^{-1} \text{ cm}^{-1}$), 516 nm ($\epsilon = 837 \text{ M}^{-1} \text{ cm}^{-1}$) and 490 nm ($\epsilon = 710 \text{ M}^{-1} \text{ cm}^{-1}$), 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 $\log K_f$ (1 M, 25 °C) ranging from 15.8 for the monosalicylate-Fe(III) complex to 35.3 for the trisalicylate-Fe(III) complex (Martell & Smith, 1977).

The UV-visible absorption spectra of compounds A_1 and A_2 were determined under the same solvent conditions described above. The results obtained (spectrum A in Fig. 2*a*) indicate that compounds A_1 and

A_2 correspond to pyochelin, since each showed typical absorption maxima at 250 and 310 nm, as previously reported by Cox & Graham (1979). Moreover, compounds A_1 and A_2 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 fluorimetric 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 A_1 , A_2 and B were compared for all the strains examined. Each compound appeared to be homogeneous as no difference was observed among compounds A_1 , A_2 and B extracted from the different *Pseudomonas* strains. The possibility that compound B (salicylic acid) could originate from spontaneous degradation of compounds A_1 and A_2 (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 CD_3OD 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 PAO1 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

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

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

Values are means ($\pm$ SD) of triplicate experiments performed with a single DCAA preparation. Fe(III) was added as 10 μ M-FeCl₃; salicylate was added either as sodium salt or as Fe(III)-complex [3:1, salicylate/Fe(III) molar ratio] at 30 μ M.

Strain	Mean generation time (min) in DCAA supplemented with:			
	Nil	Fe(III)	Salicylate	Fe(III)-salicylate
PAO1	75.2 ($\pm$ 3.6)	48.1 ($\pm$ 0.9)	68.4 ($\pm$ 2.4)	42.3 ($\pm$ 1.7)
PA46U	68.7 ($\pm$ 4.5)	43.6 ($\pm$ 2.3)	61.0 ($\pm$ 3.9)	39.8 ($\pm$ 1.1)

growth of both *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 pyoverdine and pyochelin and their ferri-forms (Visca *et al.*, 1992b).

The interesting parallels observed for the synthesis and

the functional effects of salicylate in *Pseudomonas* spp. and other bacterial genera is worth noting. Salicylic acid is a precursor of pyochelin in *P. aeruginosa* (Ankenbauer & Cox, 1988) and mycobactin in *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 *Mycobacterium* spp. (Ratledge, 1987; Ratledge & Winder, 1962; Ratledge *et al.*, 1974), *Azospirillum lipoferum* (Saxena *et al.*, 1986), *P. aeruginosa* (Meyer, 1992) and *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 *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 pyoverdine in *P. aeruginosa* (Gensberg *et al.*, 1992).

Taken together, the results presented here demonstrate that *P. aeruginosa* and *P. cepacia*, as well as *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 *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.

We are grateful to Professor V. Galeffi (Istituto Superiore di Sanità, Roma) and to Professor F. Cacace (Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive) for helpful discussions. This work was supported by a MURST grant from the project 'Control of Microbial Pathogenicity', and by a CNR grant from the targeted project 'Prevention and Control of Disease Factors'.

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