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Isolation and Characterization of *Pseudomonas aeruginosa* Mutants Requiring Salicylic Acid for Pyochelin Biosynthesis

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Pseudomonas aeruginosa **mutants requiring salicylic acid for pyochelin biosynthesis were isolated after chemical mutagenesis by plating on a siderophore detection medium. Like the wild type, these mutants incorporated 7-[^{14}C]salicylic acid into pyochelin, demonstrating that salicylic acid is an intermediate in the biosynthesis pathway of pyochelin.**

Pseudomonas aeruginosa produces two siderophores, pyoverdin and pyochelin. Pyoverdin (4, 7, 28), a hydroxamate siderophore, is a peptide containing a dihydroxyquinoline derivative characteristic of siderophores produced by fluorescent pseudomonads (10, 18, 26). Pyochelin is a structurally unique phenolate siderophore (8) which is also produced by *Pseudomonas cepacia* (22, 23) and *Pseudomonas fluorescens* (22). Pyochelin has been assigned the chemical structure 2-[2-(*o*-hydroxyphenyl)-2-thiazolin-4-yl]-3-methyl-4-thiazolidinecarboxylic acid (9).

Pyochelin plays an important role in the interaction of *P. aeruginosa* with the mammalian nutritional immunity system (6). Pyochelin has been shown to facilitate the removal of iron from the critical component of the nutritional immunity system, transferrin (25), and is also capable of stimulating bacterial growth during infections in mice (5). Mutants defective in pyochelin and pyoverdin biosynthesis are strongly inhibited by transferrin in growth assays (2). Recently, it was demonstrated that mutants with defects in ferripyochelin transport are markedly less virulent than wild-type strains of *P. aeruginosa* (23).

An earlier report (9) contained a proposed biosynthesis pathway for pyochelin which included salicylic acid as the first intermediate in the pathway. Utilizing a variation of CAS agar, a siderophore-detection medium (21), we have isolated *P. aeruginosa* mutants requiring salicylic acid for pyochelin biosynthesis.

Pyoverdin appears to be the more efficient of the two siderophores on the basis of its mobilization of iron from transferrin (2, 25), and Pvd$^+$ Pch$^-$ mutants were indistinguishable from Pvd$^+$ Pch$^+$ strains on various screening media. Therefore, it was necessary to eliminate the production of pyoverdin so that mutants in pyochelin biosynthesis could be detected. For this reason we utilized two Pvd$^-$ derivatives of *P. aeruginosa* PAO1, CDC5 (*pvd-2*) and 211-5 (*pvd-1*) (1), as parent strains.

CDC5 and 211-5 were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (12) or ethyl methanesulfonate (16) and then plated on modified CAS agar to yield 200 colonies per plate. The siderophore detection medium, CAS agar, contains a blue Fe(III)-dye complex which turns orange when the ferric ion is removed from the complex by siderophores secreted by colonies (21). CAS agar was modified to optimize the conditions required for pyochelin synthesis and mutant detection. Modification of CAS agar was necessary because of the production of pyocyanin on this medium; the blue color of pyocyanin (27) prevented the detection of orange zones in the blue background of CAS agar. The ionic control of pyocyanin biosynthesis, especially by iron and phosphate, has been well documented (11, 14). Modified CAS agar contained 5 mM KH_2PO_4 and 5 mM Na_2SO_4 in place of MM9 salts and contained 10 mM sodium succinate in place of glucose. After 24 h of incubation at 37°C, colonies lacking orange zones were picked onto modified CAS agar containing 500 μM salicylic acid. Colonies surrounded by orange zones on modified CAS agar containing salicylic acid were suspected of being mutants requiring salicylic acid for pyochelin biosynthesis. The phenotype of these mutants has been designated Sal$^-$.

Nine Sal$^-$ mutants (IA602, IA613, IA614, IA617, IA626, IA641, IA653, IA657, and IA660) were isolated. Mutants IA641 and IA653 are derivatives of strain 211-5, and the remaining mutants are derivatives of CDC5. With the exception of IA657 and IA660, which were generated by ethyl methanesulfonate mutagenesis, all mutants were derived from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutageneses. All mutants were isolated from independent mutageneses, with the exception of IA602, IA613, IA614, and IA617, which were isolated from a single mutagenesis. The Pch$^-$ phenotypes of the mutants were assayed by growing the mutants in CAA medium (7) and analyzing extracts of the culture medium by thin-layer chromatography analysis. Pyochelin on chromatograms was detected by an assay with increased sensitivity which does not depend upon iron-chelating properties. Pyochelin, normally detected on thin-layer chromatograms as green fluorescent spots under UV light which turn red when sprayed with 0.1 M $FeCl_3$–HCl, yields black spots when sprayed with ammoniacal silver nitrate reagent (15). Pyochelin contains an *N*-methylthiazolidine ring, which is known to rapidly form black silver mercaptide salts during alkaline hydrolysis in the presence of Ag^+ ion (17). As expected, all Sal$^-$ mutants were unable to produce pyochelin in the absence of salicylic acid (Fig. 1). The Sal$^-$ phenotype of these mutants was also assayed by addition of 100 μM salicylic acid to cultures. Thin-layer chromatography of culture extracts of Sal$^-$ mutants grown in the presence of salicylic acid demonstrated pyochelin synthesis by all Sal$^-$ mutants (Fig. 1). Analysis of culture extracts of two other Pch$^-$ mutants, IA1 (2) and IA607, revealed that these strains were not capable of producing pyochelin in the presence of salicylic acid. Mutants IA1 and IA607 are therefore Pch$^-$ but not Sal$^-$.

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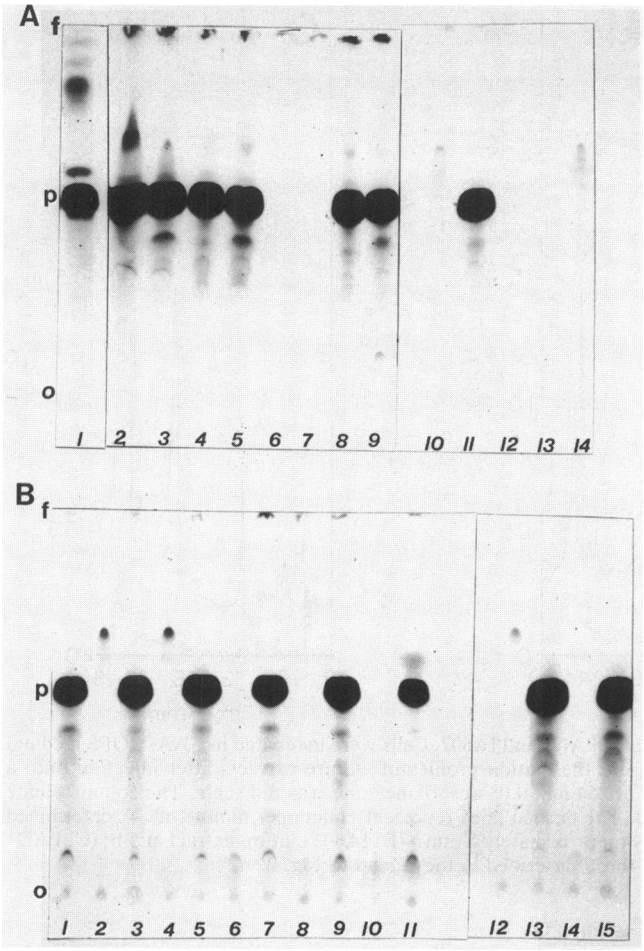

FIG. 1. Thin-layer chromatograms of acid methylene chloride extracts of *P. aeruginosa* cultures with and without 100 μM sodium salicylate. Chromatograms were developed by spraying with ammoniacal silver nitrate reagent (15). (A) Lanes: 1, pyochelin control; 2, PAO1; 3, PAO1 with salicylate; 4, CDC5; 5, CDC5 with salicylate; 6, IA1; 7, IA1 with salicylate; 8, 211-5; 9, 211-5 with salicylate; 10, IA602; 11, IA602 with salicylate; 12, IA607; 13, IA607 with salicylate; 14, IA613. (B) Lanes: 1, IA613 with salicylate; 2, IA614; 3, IA614 with salicylate; 4, IA617; 5, IA617 with salicylate; 6, IA626; 7, IA626 with salicylate; 8, IA641; 9, IA641 with salicylate; 10, IA653; 11, IA653 with salicylate; 12, IA657; 13, IA657 with salicylate; 14, IA660; 15, IA660 with salicylate. Cultures were inoculated at 10^4 CFU/ml and incubated at 37°C with shaking for 48 h. Sodium salicylate was added to cultures at inoculation. Cultures were extracted with an equal volume of methylene chloride-acetic acid (10:1). Silica gel chromatograms were developed in $CHCl_3$-ethanol-acetic acid (19:1:1). Abbreviations: f, solvent front; o, origin; p, pyochelin.

The identity of salicylic acid as an intermediate in the biosynthesis of pyochelin was demonstrated by the incorporation of radiolabeled salicylic acid into pyochelin. Cells of strain PAO1 and the Sal^- mutant IA602 grown in CAA medium were incubated at 2.24×10^{10} CFU/ml in CAA-MOPS medium (1% Casamino Acids [Difco Laboratories, Detroit, Mich.], 1 mM MOPS [morpholinopropane sulfonate], 1 mM $MgSO_4$, pH 7.1) containing 0.4 μCi of 7-[^{14}C]salicylic acid per ml. At various times during the incubation, 1-ml samples of the mixture were extracted with 2 volumes of CH_2Cl_2-acetic acid (10:1). The extracts were dried and injected onto a C18 reversed-phase high-pressure liquid chromatography (HPLC) column (Brownlee Labs, Santa Clara, Calif.). One-milliliter fractions collected from the linear, increasing methanol gradient were mixed with scintillation counting cocktail, and the presence of ^{14}C in the fractions was determined by scintillation counting (Prias; Packard, Downers Grove, Ill.). The chromatographic conditions employed in this study resulted in the elution of salicylic acid at 10.7 min and of pyochelin as two closely spaced peaks at 24.9 and 25.8 min (Fig. 2). The phenomenon of pyochelin existing as two separable forms has been observed by both HPLC and thin-layer chromatography; the two peaks have been shown to be spontaneously interconvertible and to possess the same chemical structure by nuclear magnetic resonance and mass spectrometry analysis (3).

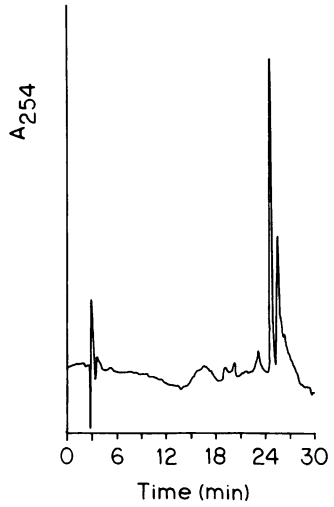

FIG. 2. HPLC separation of pyochelin into two peaks. The line tracing represents the elution profile of approximately 4 μg of pyochelin after injection onto a reversed phase (C_{18}) HPLC column. The elution profile was monitored at 254 nm; 0.08 absorbance unit was full scale. The chromatographic conditions were as follows: flow rate, 1.0 ml/min; 5-min flow of 40% methanol in 0.17 N acetic acid–0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, subsequently going to 100% methanol over 20 min.

The radioactivity at the start of the assays was associated with salicylic acid, which eluted at 10.7 min (Fig. 3A). At 5 h after the start of the assay, the radioactivity in extracts from IA602 and PAO1 was predominantly associated with the pyochelin peaks eluting at 24 to 26 min (Fig. 3B and C). There was no radioactivity associated with the salicylic acid peak at 5 h. The total amount of pyochelin produced by the Sal^- mutant, IA602, was shown by A_{254} to be much less than that of the wild-type PAO1. This was due to the ability of PAO1 to produce its own salicylic acid. However, the same amount of 7-[^{14}C]salicylic acid was incorporated into pyochelin by both strains (Fig. 3B and C). Salicylic acid was rapidly incorporated into pyochelin during the course of the reaction, as demonstrated by the total movement of the radioactivity from the [^{14}C]salicylic acid peak into the [^{14}C]pyochelin peaks by 5 h. Pch^- mutants which were not Sal^-, such as IA607, were not able to incorporate 7-[^{14}C]salicylic acid into pyochelin. Another group of pyochelin-negative mutants have been found to secrete a product identified as salicylic acid by ^{1}H nuclear magnetic resonance and mass spectrometry. We therefore conclude that salicylic acid is an intermediate in pyochelin biosynthesis.

Salicylic acid has also been reported to be incorporated

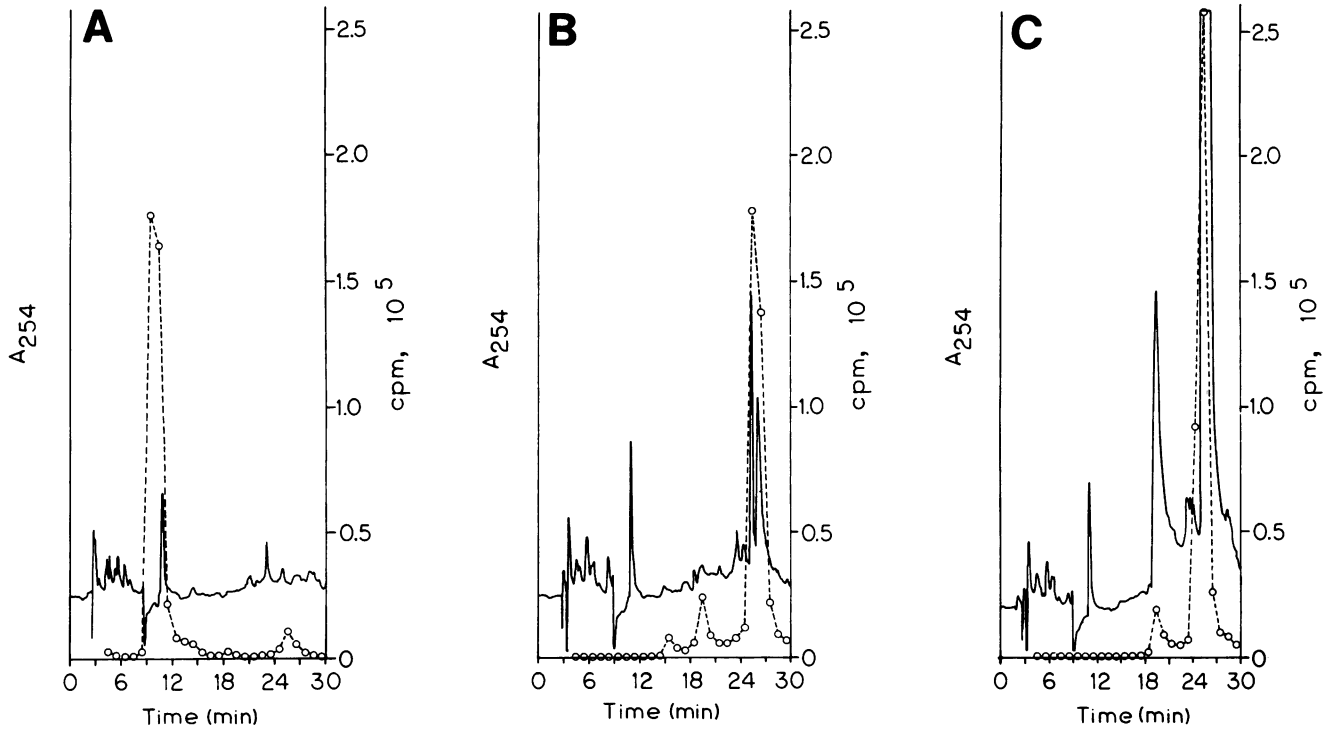

FIG. 3. Incorporation of 7-[^{14}C]salicylic acid into [^{14}C]pyochelin by strains PAO1 and IA602. Cells were incubated in CAA-MOPS medium containing 0.4 μCi of 7-[^{14}C]salicylic acid per ml. The solid lines represent the elution profiles of culture extracts after injection onto a reversed-phase (C_{18}) HPLC column. The elution profiles were monitored at 254 nm; 0.08 absorbance unit was full scale. The column eluate was fractionated into 1-ml fractions, and the ^{14}C content was determined. The dashed lines represent counts per minute of ^{14}C determined by scintillation counting. (A) IA602 culture extract at the start of the assay (approximately 2 min); (B) IA602 culture extract at 5 h; (C) PAO1 culture extract at 5 h. The chromatographic conditions were identical to those described in the legend to Fig. 2.

into mycobactin S produced by *Mycobacterium smegmatis* (13, 19, 20). In results similar to those of this study, Ratledge and Hall (19) isolated mycobactin-negative mutants of *M. smegmatis* which required salicylic acid for mycobactin synthesis and incorporated [^{14}C]salicylic acid into [^{14}C] mycobactin.

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