Identification of NRRL strain B-18602 (PR3) as *Pseudomonas aeruginosa* and effect of phenazine 1-carboxylic acid formation on 7,10-dihydroxy-8(*E*)-octadecenoic acid accumulation†

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A new compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD), produced from oleic acid by a new bacterial isolate PR3, was discovered in 1991. We have now identified isolate PR3 as a strain of *Pseudomonas aeruginosa* by DNA reassociation studies. Strain PR3 also produced a crystalline yellowish compound the structure of which, as determined by GC/MS and NMR, is phenazine 1-carboxylic acid (PCA). In cultures of PR3, high PCA production was associated with low DOD accumulation.

Key words: Dihydroxy fatty acid, phenazine 1-carboxylic acid, *Pseudomonas aeruginosa*.

In our continuing screening programme for new industrial chemicals produced from vegetable oils by biotechnology, we discovered that a new bacterial strain, PR3, isolated from a water sample taken at a pig farm in Morton, Illinois, converted oleic acid to a new compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) (Hou *et al.* 1991). The reaction is unique in that it involves a hydroxylation at two positions and a rearrangement of the double bond of the substrate molecule. An intermediate in the production of DOD, 10-hydroxy-8-(Z)-octadecenoic acid (HOD), was also found (Hou & Bagby 1992). Isolate PR3 is a motile, small rod-shaped, Gram-negative bacterium. It has multiple polar flagella and is oxidase positive. Based on these observations, isolate PR3 was tentatively assigned to the genus *Pseudomonas* (Hou & Bagby 1991). We then screened 20 *Pseudomonas* strains in our Agricultural Research Service (ARS) Culture Collection for their ability to convert oleic

acid to DOD and found none were positive. Recently, we isolated a yellowish compound from the reaction mixture and observed that when strain PR3 produced more of this yellow compound, its activity to convert oleic acid to DOD decreased. In light of these findings, we decided to conduct further identification of isolate PR3 and study the relationship between the production of the yellow compound and the oleic acid hydroxylation activities. This paper describes the identification of the bacterial isolate and the nature of the yellow compound.

Materials and Methods

Microorganisms
Microbial cultures were obtained from the ARS Culture Collection except for bacterial isolate PR3 which was isolated from a water sample from a pig farm in Morton, Illinois. Each culture was grown at 30°C aerobically in a Fernbach flask (shaken at 200 rev/min) containing 1 l of screening medium containing (g/l): glucose, 4; $(NH_4)_2HPO_4$, 10; K_2HPO_4, 2; yeast extract, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.014; and nicotinic acid, 0.1. The pH of the medium was adjusted to 7.0 with dilute phosphoric acid.

To a 24-h-old culture in shake flasks, an aliquot amount of oleic acid (7 g) was added and the flasks were shaken again at 200 rev/min at 30°C for another 2 days.

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Chemicals

Oleic acid (>99% purity) was purchased from Nu-Check-Prep (Elysian, MN). All other chemicals were reagent grade and were used without further purification. Thin-layer precoated Kieselgel 60 F_{254} plates were obtained from EM Science (Cherry Hill, NJ).

DNA Isolation and Reassociation and G + C Ratios

For DNA extraction, cells were grown for 18 h in tryptone/glucose/yeast extract broth (Haynes *et al.* 1955) at 28°C on a rotary shaker at 200 rev/min. DNA was extracted and purified by a modification of the method of Marmur (1961). After the ribonuclease and chloroform/iso-amyl alcohol (24:1 v/v) treatments described by Marmur (1961), the crude DNA was purified by gradient centrifugation in CsCl as described by Maniatis *et al.* (1982).

The G + C content was estimated by the thermal melt procedure described by Mandel & Marmur (1968). *Escherichia coli* DNA with a G + C content of 51 mol% was used for comparison.

In preparation for the renaturation studies, native double-stranded DNA was sheared by two passages through a French pressure cell at 35 MPa. After filtration through a membrane filter (pore size 0.45 μm), the sheared DNA solution was dialysed exhaustively against a 0.001 M EDTA solution containing 5 ml of 1 × SSC (0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0)/l and subsequently freeze dried. The dried material was dissolved in 2 ml double-distilled water.

The kinetics of DNA renaturation was measured in reaction mixtures, each containing 0.30 ml of 10 × SSC, 0.20 ml DMSO, approximately 70 μg of DNA, and double-distilled water to bring the volume to 1 ml. In reaction mixtures containing two different DNA, the quantities (about 35 μg) of the nucleic acids were adjusted to differ by no more than 0.15 μg. The reaction mixtures were each placed in 0.4 ml quartz cuvettes that were then sealed with teflon-lined stoppers. Denaturation and renaturation were effected and monitored with a Gilford UV-VIS thermal analyser system. A denaturation temperature of 90°C was reached rapidly and was held for 10 min after the hyperchromic shift at 260 nm. Subsequently, the temperature was lowered to 70°C; the optical density at this point was taken to represent 0% renaturation (although some reassociation undoubtedly occurred). The absorbance change at 260 nm was monitored for 30 min, during which the rate of renaturation was apparently linear, even though second-order kinetics was displayed over a longer period of time. All determinations were repeated three times. Reassociation values were calculated using the equation of De Ley *et al.* (1970).

Analytical Methods

At the end of the incubation, the culture broth was acidified and extracted with an equal volume of ethyl acetate followed by an equal volume of diethyl ether. The solvents were removed from the combined extracts with a rotary evaporator. The reaction products were assayed by TLC and GC according to Hou & Bagby (1991). For TLC, toluene/dioxane/acetic acid (79:14:7 by vol) was used. The chromatograms were visualized first by vapour and then by spraying the plate with 50% sulphuric acid and heating at 100°C for 10 min. For GC, the sample was methylated with diazomethane. The methyl esters were chromatographed with a Supelco SPB^{T-1} capillary column (15 m × 0.32 mm), 0.25 μm thickness, run isothermally at 200°C. Eluants were integrated electronically. For quantitative analysis, palmitic acid was added as an internal standard prior to the solvent extraction. A linear relationship was established on the peak area ratios of product versus methyl palmitate.

HPLC was conducted with a Dynamax-60A silica column (25 cm × 21.4 mm) using as solvent hexane/ethyl acetate/

trifluoroacetic acid (80:20:1 by vol), at a flow rate of 4 ml/min. The eluent was monitored with a refractive index detector and spectrophotometrically.

Electron impact mass spectra were obtained with a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 Series Mass Selective Detector. Proton and C-13 nuclear magnetic resonance spectra were determined in deuterated chloroform with a Bruker WM-300 spectrometer, operating at a frequency of 300 MHz and 75.5 MHz, respectively. UV/Vis absorption spectra were recorded with a Beckman DU8 spectrophotometer (Fullerton, CA).

Isolation of the Yellow Compound

The combined solvent extracts of the reaction products were evaporated to dryness. The solid material (2.2 g) collected from a 1-l flask was washed twice with 50 ml of hexane to remove the major portion of the unreacted substrate i.e. oleic acid. The remaining solid materials were dissolved in a small amount of methylene chloride and were chromatographed on a silica gel 60 column (30 × 2.2 cm) which was pre-equilibrated with methylene chloride. The column was washed with 250 ml methylene chloride and then eluted with: (1) a 300-ml mixture of methylene chloride/methanol (98:2 by vol); and (2) a 300-ml mixture of methylene chloride/methanol (95:10 by vol). The fraction collector was started after the column washing. Portions (6 ml) were collected and analysed by TLC. Tube numbers 8 to 20 (400 mg) mainly contained the yellowish compound. Tube numbers 35 to 45 were mainly HOD and tube numbers 55 to 90 were DOD. The yellow compound fraction was subjected to HPLC. Portions (4 ml) were collected and analysed by TLC. The yellow compound was eluted at a retention time of 17 min (Figure 1). The fraction (180 mg) appeared as a single spot (Figure 2, $R_f = 0.38$) on TLC analysis. GC of methylated materials showed one peak (97% purity) at a retention time of 13 min.

Results and Discussion

Identification of the Yellow Compound

In the GC/MS analysis, the electron impact (EI) mass spectrum of the methylated yellow compound showed a molecular ion at m/e 238 and a base peak at 180 (Figure 3). The peak at 223 represents loss of the methyl group;
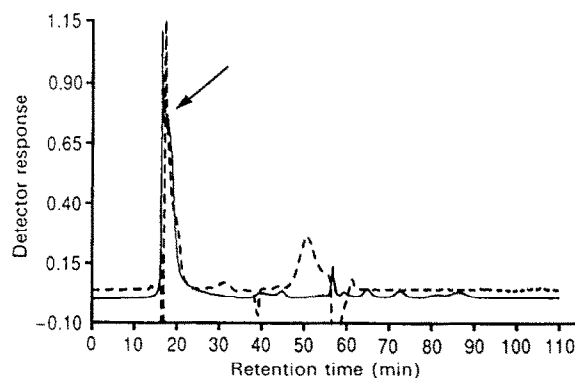

Figure 1. HPLC of the yellow compound produced by isolate PR3. The conditions are described in Materials and Methods. ————Refractive index; ————UV. Arrow indicates yellow compound fraction.
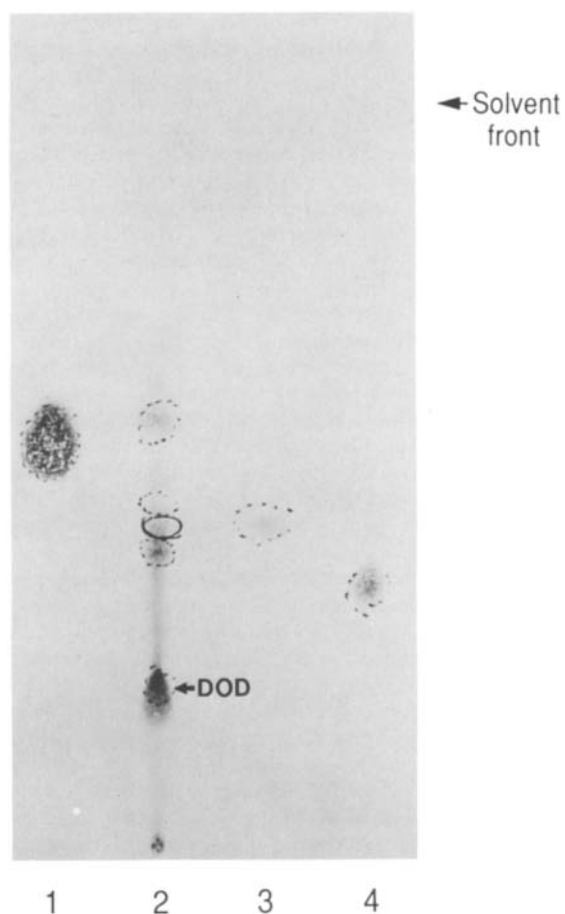
Figure 2. Thin-layer chromatography of PCA using toluene/dioxane/acetic acid (79:14:7, by vol) as solvent. Spots circled with a broken line were visualized with vapour. DOD—7,10-Dihydroxy-8(E)-octadecenoic acid; lane 1—oleic acid; lane 2—48 h reaction products; lane 3—purified phenazine 1-carboxylic acid (PCA); lane 4—10-hydroxy-8(Z)-octadecenoic acid (HOD).

the peak at 179 represents loss of the carboxyl group. The base peak at 180 reflects the tendency of the m/e 179 moiety to capture a hydrogen to complete the phenazine nucleus. These mass spectra are identical to those of phenazine 1-carboxylic acid (Toohey et al. 1965).

The yellow compound was also subjected to proton and ^{13}C-NMR analyses. Both the proton and ^{13}C-NMR data
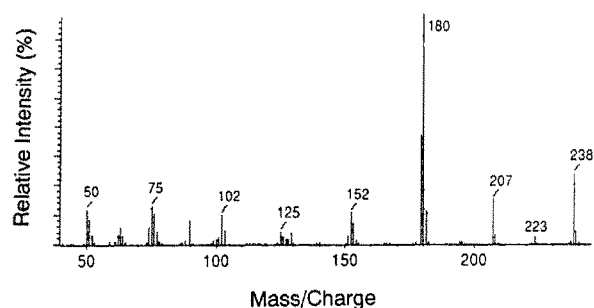

Mass/Charge

Figure 3. EI mass spectrum of the methylated yellow compound.

agreed well with those reported for phenazine 1-carboxylic acid (Romer 1982; Gurusiddaiah et al. 1986).

UV/Vis spectra of the yellow compound in methylene chloride showed absorption maxima at 369 nm ($\varepsilon = 5.09 \times 10^3$) and 251 nm ($\varepsilon = 5.41 \times 10^3$). The melting point of the compound was 239°C. All of these data indicate that the yellow compound is phenazine 1-carboxylic acid (PCA).

The fluorescent Pseudomonas P. aureofaciens (Haynes et al. 1955), P. aeruginosa (Takeda 1958; Chang & Blackwood 1969) and P. fluorescens (Brisbane et al. 1987) are known to produce phenazine pigments. The multiple polar flagella of strain PR3 (Hou & Bagby 1991) differ from the monotrichous flagella described for P. aeruginosa (Palleroni 1984). Therefore, strain PR3 was subjected to further identification.

Identification of Strain PR3

The present study showed that PR3 produced fluorescein on King's medium B as well as pyocyanin on King's medium A (King et al. 1954), suggesting that the organism was a strain of P. aeruginosa.

DNA reassociation measurements were carried out to confirm the identity of PR3. Whereas PR3 yielded high DNA reassociation values (100%) with the type strain of Pseudomonas aeruginosa, it gave low values of 27.0 to 45.4% with the type strains of P. aureofaciens, P. chlororaphis and P. fluorescens (Table 1). Based on a thermal melting value of 96.6°C determined for PR3 DNA, the G + C value was estimated to be 66.5 mol%, a value almost identical to that of the P. aeruginosa type strain.

Based on these observations, it was concluded that PR3 is a strain of P. aeruginosa and it has been deposited in the ARS Culture Collection as P. aeruginosa NRRL B-18602.

Relationship between PCA and DOD Synthesis

PR3 was originally isolated for its ability to convert oleic acid to DOD. Recently, we found that the production of DOD may be affected by PCA synthesis. Experiments were conducted to determine the relationship between the production of the two compounds. Cultures were grown in 1 l volumes of either screening medium or TGY broth in

Table 1. DNA relatedness of PR3 and type strains of selected *Pseudomonas* species.

Type strain	G + C content* (mol %)	DNA reassociation with PR3 (%)
P. aureofaciens NRRL B-560	64	45.4
P. aeruginosa NRRL B-770	67	100.0
P. chlororaphis NRRL B-1576	64	27.3
P. fluorescens NRRL B-14678	61	34.0

* Taken from Palleroni (1984). The G + C content of PR3, determined by thermal melt, was 66.5 mol%.

Table 2. Relationship between the production of PCA and DOD by isolate PR3 in the presence and absence of oleic acid.

Growth medium	Products (mg/l)	
	PCA	DOD
Without oleic acid:		
TGY broth	90	0
Screening medium	120	0
With oleic acid:		
TGY broth + oleic acid	30	140
Screening medium + oleic acid	20	450

PCA—phenazine 1-carboxylic acid; DOD—7,10-dihydroxy-8(*E*)-octadecenoic acid.

Fernbach flasks and incubated at 30°C on a rotary shaker at 200 rev/min for 24 h. After 24 h, each of the cultures was supplemented with 8 ml (7 g) of oleic acid and then incubation at 30°C on the shaker was resumed. Unsupplemented controls were also maintained.

The results in Table 2 show that PCA (about 100 mg/l) normally formed by strain PR3 in the absence of oleic acid. The addition of oleic acid to the medium depressed PCA production and promoted that of DOD. The relationship between the production of PCA and the synthesis of DOD is not clear. However, two general explanations for these observations are possible. One is that the syntheses of the substances are directly or indirectly linked in such a way that any commitment to the synthesis of one compound occurs at the expense of the other. The alternative explanation is that conditions are produced in the growth medium that are favourable to the synthesis of one compound and not the other. Similar variabilities in the production of phenazine compounds by fluorescent *Pseudomonas* have been reported (Kanner *et al.* 1978). Gurusiddaiah *et al.* (1986) found that the yield of phenazine antibiotic from *Pseudomonas* spp. varied sharply between 0 and 600 mg/l within the same batch of medium. They suggested that an unknown factor is probably necessary to trigger the production of the yellow compound in the medium. Nevertheless, it seems clear that only when production of PCA was depressed did strain PR3 promote the synthesis of DOD. Currently, we are trying to stabilize and maximize the ability of strain PR3 to transform oleic acid to DOD.

Acknowledgements

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