Identification of phenyldecanoic acid as a constituent of triacylglycerols and wax ester produced by *Rhodococcus opacus* PD630

Héctor M. Alvarez,¹ Heinrich Luftmann,² Roxana A. Silva,¹ Ana C. Cesari,¹ Alberto Viale,³ Marc Wältermann⁴ and Alexander Steinbüchel⁴

Author for correspondence: Héctor M. Alvarez. Tel: +54 297 4550 339. Fax: +54 297 4550 339.
e-mail: halvarez@unpata.edu.ar

¹ Departamento de Bioquímica, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, CC 1078, Km 4, 9000 Comodoro Rivadavia, Chubut, Argentina

² Institut für Organische Chemie der Westfälischen Wilhelms-Universität Münster, Corrensstraße 40, D-48149 Münster, Germany

³ Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Buenos Aires, Buenos Aires, Argentina

⁴ Institut für Mikrobiologie der Westfälischen Wilhelms-Universität Münster, Corrensstraße 3, D-48149 Münster, Germany

Phenyldecane supported growth and lipid accumulation of *Rhodococcus opacus* PD630 during cultivation under nitrogen-limiting conditions. The results of this study suggested that the hydrocarbon phenyldecane was degraded by monoterminal oxidation, followed by β -oxidation of the alkyl side-chain to phenylacetic acid, and by an additional degradative route for the oxidation of the latter to intermediates of the central metabolism. α -Oxidation of phenyldecanoic acid also occurred to some extent. Phenyldecanoic acid, the monoterminal oxidation product, was also utilized for the biosynthesis of a novel wax ester and novel triacylglycerols. The formation of the wax ester phenyldecylphenyldecanoate probably resulted from the condensation of phenyldecanoic acid and phenyldecanol, which were produced as metabolites during the catabolism of phenyldecane. Two types of triacylglycerol were detected in phenyldecane-grown cells of strain PD630. Triacylglycerols containing only odd- and even-numbered aliphatic fatty acids, as well as triacylglycerols in which one fatty acid was replaced by a phenyldecanoic acid residue, occurred. Other phenyl intermediates, such as phenylacetic acid, phenylpropionic acid, 4-hydroxyphenylpropionic acid, protocatechuate and homogentisic acid, were excreted into the medium during cultivation on phenyldecane. On the basis of the results obtained, pathways for the catabolism and assimilation of phenyldecane by *R. opacus* PD630 are discussed.

Keywords: *Rhodococcus opacus* PD630, phenyldecane, triacylglycerol, wax ester, phenyldecylphenyldecanoate

INTRODUCTION

Phenylalkanes consisting of a phenyl group substituted with an alkyl residue of variable chain length are common components of crude oil and detergents (Sikkema *et al.*, 1995). These compounds offer two alternative pathways for degradation: (1) oxidative attack at the aromatic ring, resulting in the formation of alkyl-catechols, and (2) oxidation of the alkyl side-chain, resulting in the formation of aromatic fatty acids. These compounds are then oxidized to dihydroxylated aromatic derivatives, which are further degraded by ring fission (Gibson & Subramanian, 1984). The oxidation

of phenylalkanes and phenylalkanoic acids has been reported for different bacteria, such as species belonging to the genera *Nocardia* (Sariaslani *et al.*, 1974), *Rhodococcus* (Warhurst & Fewson, 1994; Alvarez *et al.*, 1996) and *Pseudomonas* (García *et al.*, 1999). According to García *et al.* (1999), all bacteria able to assimilate phenylacetic acid or benzoic acid via a CoA intermediate would be able to utilize phenylalkanoic acids as sole carbon source, since phenylacetic acid is a β -oxidation product from the biodegradation of phenylalkanes possessing an alkyl side-chain with an even number of carbon atoms.

Phenyldecane-grown cells of *Rhodococcus opacus* PD630, which were also able to catabolize phenylacetic acid, intracellularly accumulated oxidation products of the substrate, with phenyldecanoic acid as the major

Abbreviations: ESI-MS, electron spray ionization mass spectrometry; TAG, triacylglycerol.

compound and also lesser amounts of phenyloctanoic, phenylhexanoic, phenylnonanoic acids and diacylglycerols (Alvarez *et al.*, 1996). Members of the genus *Rhodococcus* are widely distributed in soil environments. These micro-organisms show a broad capacity and metabolic spectrum for the biodegradation of different kinds of pollutants, such as hydrocarbons, herbicides or other xenobiotic compounds (Finnerty, 1992; Warhust & Fewson, 1994). In addition, recent studies demonstrated that these bacteria are able to accumulate triacylglycerols (TAGs) from different carbon sources, including hydrocarbons, during cultivation under nitrogen-starvation conditions (Alvarez *et al.*, 1996, 1997, 2000). In this context, the biodegradation and production of cellular lipids by *Nocardia globerula* 432 from the recalcitrant branched hydrocarbon pristane under conditions restricting growth has been reported recently (Alvarez *et al.*, 2001). These results suggest that these Gram-positive bacteria may be employed to eliminate pollutants from the environment under a broad range of metabolic and environmental conditions. Several reports considered the potential of these bacteria for *in situ* bioremediation of contaminated environments (Yakimov *et al.*, 1999; Wagner-Dobler *et al.*, 1998; White *et al.*, 1998).

On account of the ability of *R. opacus* PD630 to accumulate metabolites and lipids under unbalanced growth conditions, we investigated in this study the catabolism and assimilation of derivatives of phenyldecane in this strain.

METHODS

Bacterial strain, media and growth conditions. *R. opacus* strain PD630 (DSM 44193) was isolated and characterized in a previous study (Alvarez *et al.*, 1996). Cells were grown aerobically at 25 °C in nutrient broth medium (0.8%, w/v) or in mineral salts medium, according to Schlegel *et al.* (1961), with the carbon source indicated in the text. To promote accumulation of lipids, the concentration of ammonium chloride in the mineral salts medium was reduced to 0.05 g l⁻¹. Solidified media were prepared by adding 1.8% (w/v) agar. Phenyldecane was obtained from Sigma.

Growth was monitored by noting the occurrence of visible growth (turbidity) in the culture medium, using a Klett-Summerson photometer and a filter at a wavelength of 436 nm.

Induction procedure. Induction studies based on measuring the time-dependent release of CO₂ due to the mineralization of the carbon source were carried out as described by Frederikson *et al.* (1991). For this purpose, the cells were grown at 25 °C on mineral salts medium agar plates containing 0.3 ml of the respective carbon source on a filter-paper disc in the lid or containing 1% (w/v) sodium gluconate in the agar. After 48 h incubation, the cells were harvested from the plates, washed twice with sterile NaCl solution (0.85%, w/v) and subsequently resuspended to an optical density at 436 nm of 2.5 in 500 ml flasks containing 50 ml mineral salts medium plus the hydrocarbon as sole carbon source. Chloramphenicol (200 µg ml⁻¹) was also added to prevent further protein synthesis. Controls without chloramphenicol were done under identical conditions. Each flask was equipped with a vial containing 2 ml 1 M NaOH to absorb CO₂ produced by the

cells; these vials were exchanged every 24 h with new vials containing fresh NaOH solution during the time course of the experiment. The flasks were tightly sealed with wrapped rubber stoppers and incubated for 24 h at 25 °C on a rotary shaker at 120 r.p.m. CO₂ production was monitored by titration with 1 M HCl.

TLC of extracted aromatic compounds and lipids. Aromatic compounds were extracted from the supernatants with methanol and were subjected to TLC on silica-gel 60F254 plates (Merck), applying chloroform/acetic acid (9:1, v/v) as the solvent system. Compounds were visualized under UV light and identified by comparison of their *R_F* values with those of phenylacetic acid, phenylpropionic acid, homogentisic acid, 4-hydroxyphenylpropionic acid, DL-mandelic acid (Sigma) and 4-hydroxybenzoic acid (Merck), which were used as reference substances.

For identification of lipids, whole cells were extracted with a mixture of chloroform and methanol (2:1, v/v), and the extracts were separated by TLC, which was performed on silica-gel 60F254 plates (Merck) using a mixture of hexane, diethyl ether and acetic acid (80:20:1, by vol.) as the solvent system. Lipid fractions were visualized after brief exposure to iodine vapour. Palmitic acid, stearic acid, dipalmitoylglycerol, tripalmitoylglycerol and cetylpalmitate (Merck) were used as reference substances.

Qualitative determination of intermediates in supernatants.

Culture supernatants obtained by centrifugation were analysed for excreted intermediates by liquid chromatography, using HPLC apparatus (Knauer). Separation was achieved by reversed-phase chromatography on Nucleosil-100 C₁₈ (5 µm particle size, 250 mm × 4.0 mm column) with a gradient of 0.1% (v/v) formic acid (eluent A) and acetonitrile (eluent B) in a range of 20–100% (v/v) eluent B and at a flow rate of 1 ml min⁻¹. The compounds were identified by their retention times and the corresponding spectra were obtained with a diode array detector (WellChrom Diodenarray-Detektor K-2150; Knauer) (Priefert *et al.*, 1997).

Analysis of neutral lipids and fatty acids. To determine the fatty acid content of the cells and the composition of lipids, 3–5 mg lyophilized whole cells or the triacylglycerol fraction obtained from preparative TLC were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid. The resulting fatty acid methyl esters were analysed by GC on a Konik HRGC3000 gas chromatograph equipped with an Innovax capillary column (30 m × 0.53 mm) and a flame-ionization detector (Brandl *et al.*, 1988; Alvarez *et al.*, 1996). A 2 µl portion of the organic phase was analysed after split injection (split ratio 1:20), and nitrogen was used as the carrier gas at a flow rate of 50 ml min⁻¹. The temperature of the injector and the detector was 260 °C, whereas a temperature programme was used for efficient separation of the methyl esters on the column (150 °C for 1 min, temperature increases of 5 °C min⁻¹, 240 °C for 10 min).

Electron spray ionization mass spectrometry and tandem electron spray ionization mass spectrometry. Electron spray ionization mass spectrometry (ESI-MS) and tandem ESI-MS/MS experiments were conducted with a Micromass type Quattro LCZ (Beverly) quadrupole mass spectrometer. In MS/MS mode, fragmentation was achieved by introducing argon in the reaction chamber in front of the second quadrupole. The lipid samples were directly transferred from TLC plates with a mixture of chloroform and methanol (5:1, v/v; flow rate 100 µl min⁻¹) into the ESI source using a TLC plate elution probe (DGMS 2001, P31, constructed by

H. Luftmann, Institut für Organische Chemie, Westfälische Wilhelms-Universität, Münster, Germany).

RESULTS

Excretion of metabolites during cultivation of *R. opacus* on phenyldecane

The occurrence of extracellular metabolites during the cultivation of cells of *R. opacus* strain PD630 on phenyldecane was analysed by HPLC and TLC and the compounds were identified according to the R_t or R_F values, respectively, in comparison with reference compounds. Three classes of compounds were detected: (1) phenylalkanoic acids with short-chain-length alkyl substituents, (2) monohydroxylated phenyl compounds, and (3) dihydroxylated phenyl substances. The metabolites isolated from the supernatants included the following: phenylacetic acid (R_t 11.06 and R_F 0.79), phenylpropionic acid (R_t 11.30 and R_F 0.79), 4-hydroxyphenylpropionic acid (R_t 5.91 and R_F 0.64), protocatechuate (R_t 3.70 and R_F 0.50) and homogentisic acid (2,5-dihydroxyphenylacetic acid) (R_t 2.92 and R_F 0.28), in HPLC and TLC analysis, respectively. The main compounds were homogentisic acid, 4-hydroxyphenylpropionic acid and phenylacetic acid, whereas phenylpropionic acid and protocatechuate occurred as minor compounds. Other intermediates, which appeared as minor compounds after TLC analysis and which exhibited R_F values of 0.19, 0.23, 0.38 and 0.44, remained unidentified. Growth experiments demonstrated that *R. opacus* PD630 was able to use the identified metabolites as sole carbon and energy source for growth (not shown).

Analysis of intracellular metabolites accumulated by *R. opacus* during growth on phenyldecane

Cells of strain PD630 accumulated neutral lipids during cultivation on phenyldecane as sole carbon source under nitrogen-limiting conditions. These lipids were separated by TLC analysis and identified by comparison of their R_F values with those of reference substances and by additional chemical analysis. After 4 d cultivation of the cells on phenyldecane, four spots of lipids were detected on the TLC plates (data not shown). The spot exhibiting an R_F value of 0.13 contained the diacylglycerol fraction, as revealed by comparison with the respective R_F value of a dipalmitoylglycerol standard. The occurrence of diacylglycerols in phenyldecane-grown cells of strain PD630 has been reported previously (Alvarez *et al.*, 1996). In addition, detailed analysis of the spots occurring in TLC in a previous study confirmed the occurrence of diacylglycerols in cells of *R. opacus* PD630 (Wältermann *et al.*, 2000). The other compounds were identified by ESI-MS and ESI-MS/MS after separation on TLC plates. The spot exhibiting an R_F value of 0.43 contained a mixture of TAGs with pseudomolecular ions between m/z $[M + Na]^+$ 850 and 950. The mass spectrum obtained was very similar to that of TAGs accumulated by strain PD630 during cultivation

Fig. 1. ESI-MS spectrum of TAGs isolated from cells of *R. opacus* strain PD630 cultivated on phenyldecane under nitrogen-starvation conditions after TLC separation. TAGs exhibiting an R_F value of 0.54 were isolated from a TLC plate and analysed. (a) Pseudomolecular ions of whole TAGs each containing one phenyldecanoic acid residue. (b) ESI-MS/MS spectrum of the main pseudomolecular ion with m/z 847 and the corresponding fragmentation pattern. All assignments correspond to $[M + Na]^+$.

on gluconate (Wältermann *et al.*, 2000). These TAGs contained odd- and even-numbered aliphatic fatty acids with carbon chain lengths ranging from 13 to 19 carbon atoms, palmitic acid, margaric acid, *cis* Δ^9 -heptadecenoic acid and oleic acid being major components (Alvarez *et al.*, 1996; Wältermann *et al.*, 2000). An ESI mass spectrum of the purified TLC fraction exhibiting an R_F value 0.54 revealed pseudomolecular ions in the range, for m/z $[M + Na]^+$, 800 to 880 (Fig. 1a). The main ions represented a mixture of TAGs in which one acyl group was replaced by a phenyldecanoic acid residue. The occurrence of fatty acids with a phenyl group in the TAG of this TLC fraction was confirmed by ESI-MS/MS analysis. One ESI-MS/MS spectrum and the fragmentation pattern of the pseudomolecular ion with an m/z $[M + Na]^+$ of 847 are presented in Fig. 1(b) as an example. The spectrum showed the occurrence of odd-numbered fatty acids among the TAGs, since the mass differences between ionic groups frequently amounted to 14. Similar results were previously reported by Wältermann *et al.* (2000) for TAGs accumulated by gluconate-grown cells of *R. opacus* PD630.

Fig. 2. ESI-MS/MS mass spectrum and fragmentation pattern of the aromatic wax ester phenyldecylphenyldecanoate accumulated by *R. opacus* strain PD630 during growth on phenyldecane. The compounds exhibiting an R_F value of 0.88 were isolated from a TLC plate and analysed. All assignments correspond to $[M + Na]^+$.

Finally, the compound exhibiting an R_F value of 0.88 was identified as the wax ester phenyldecylphenyldecanoate. Its ESI-MS/MS spectrum and the fragmentation pattern are shown in Fig. 2. Interestingly, other wax esters, consisting, for example, of phenyldecanoic acid and decanoic acid, could not be detected.

Regulation of phenyldecane catabolism

To reveal which enzymes are responsible for the oxidation of phenyldecane, and to evaluate whether they are constitutively expressed or induced, two-stage cultivation experiments with a different substrate in each stage, in the presence of chloramphenicol in the second stage, were done with cells of strain PD630. The amounts of CO_2 formed from the carbon source in the second cultivation stage were measured. The results of these mineralization experiments are shown in Table 1. Degradation of phenyldecane was clearly an inducible process. When the cells were grown on gluconate, phenylacetic acid or phenylalanine in the first stage, they were unable to degrade phenyldecane during cultivation in the second stage. In contrast, phenyldecane itself and hexadecane induced mineralization of phenyldecane. In addition, the exposure of cells with phenyldecane, phenylacetic acid or phenylalanine induced the catabolism of phenylacetic acid. Gluconate also induced the mineralization of phenylacetic acid to some extent, probably because this last compound may be a degradation intermediate of aromatic amino acids generated in gluconate-grown cells of *R. opacus* PD630.

DISCUSSION

This study revealed the formation of hitherto undescribed wax esters and TAGs containing phenyldecanoic acid as constituents in cells of *R. opacus* strain PD630 during cultivation of the cells on phenyldecane. On the basis of the results of this study, three steps for the oxidation of phenyldecane by *R. opacus*

Table 1. Mineralization of phenyldecane and phenylacetic acid by cells of *R. opacus* strain PD630 in the presence of chloramphenicol after previous growth of cells on various substrates

In the first stage, cells were grown on the carbon source mentioned in the first column. The cells were then resuspended in fresh mineral salts medium containing the carbon source mentioned in the second column plus chloramphenicol and incubated for a further 24 h (second stage). The amounts of CO_2 released during this period were recorded.

Substrate of the first stage	Substrate of the second stage	CO_2 (mmol)
Gluconate	Phenyldecane	0
Phenyldecane	Phenylacetic acid	0.036
Phenyldecane	Hexadecane	0.036
Phenyldecane	Phenyldecane	0.055
Gluconate	Hexadecane	0.018
Hexadecane	Phenyldecane	0.073
Hexadecane	Hexadecane	0.073
Gluconate	Phenylbutyric acid	0
Gluconate	Phenylacetic acid	0.073
Phenylacetic acid	Phenyldecane	0
Phenylacetic acid	Phenylbutyric acid	0.019
Phenylacetic acid	Phenylacetic acid	0.165
Phenylalanine	Hexadecane	0.018
Phenylalanine	Phenyldecane	0.018
Phenylalanine	Phenylacetic acid	0.218

PD630 can be distinguished. (1) The induction of phenyldecane degradation by hexadecane suggests that probably both phenylalkanes and n-alkanes are oxidized by the same enzymes. Alkane monooxygenase, alcohol dehydrogenase and aldehyde dehydrogenase probably catalyse the monoterminal oxidation of both compounds. (2) The resulting phenyldecanoic acid is then further degraded mainly by the β -oxidation of the alkyl side-chain to phenylacetic acid. The occurrence of this catabolic route for the oxidation of phenylalkanes was previously reported for other actinomycetes (Gibson & Subramanian, 1984; Warhurst & Fewson, 1994). In addition, α -oxidation must also occur to some extent, as indicated by the presence of phenylnonanoic acid and phenylpropionic acid in the cells and in the supernatants. (3) The α - and β -oxidation products, phenylpropionic and phenylacetic acid, respectively, are probably hydroxylated prior to cleavage of the aromatic ring, which results in the formation of linear intermediates that are further converted to central intermediates. These results suggest that phenylacetic acid is, like phenylalanine, catabolized via homogentisic acid (Olivera *et al.*, 1998), because the capacity to degrade phenylacetic acid was induced in phenylalanine- and also in phenyldecane-grown cells of *R. opacus* strain PD630. The results of this study demonstrated that strain PD630 possesses the necessary enzymes for the aromatic ring fission of phenylacetic acid; however, these enzymes were not able to attack the aromatic ring of phenyldecane. This suggested that the occurrence of an alkyl side-chain in the aromatic molecule may hinder the oxidative attack by strain PD630.

However, phenyldecanoic acid is directed not only to catabolic pathways (α - and β -oxidation) in *R. opacus* PD630 but also towards anabolic routes, such as the biosynthesis of a novel wax ester and novel TAGs. In contrast, it is very interesting that (though it remains unclear why) wax esters composed of regular fatty acids and the corresponding alcohols were not synthesized when *R. opacus* PD630 was grown on gluconate under nitrogen starvation (Wälfertmann *et al.*, 2000). To our knowledge, this is the first report on the formation of waxes and TAGs containing aromatic constituents in bacteria. In this context, the accumulation of unusual polyhydroxyalkanoates bearing a phenyl group by *Pseudomonas* species has to be mentioned (Fritzsche *et al.*, 1990; García *et al.*, 1999). The synthesis of such polyhydroxyalkanoates required the occurrence of a polyhydroxyalkanoate synthase with broad substrate specificity. In contrast, *R. opacus* PD630 is unable to store 3-hydroxyalkanoic acids as polyhydroxyalkanoates but accumulates fatty acids as neutral lipids under the culture conditions in this study (Alvarez *et al.*, 1996).

Whether or not the formation of waxes and TAGs with aromatic constituents in *R. opacus* is physiologically relevant remains to be evaluated. As result of its broad metabolic capacity, unusual fatty acids such as phenyldecanoic acid may be generated from phenylalkanes, which may disturb the membrane fluidity if they are

incorporated into phospholipids. Therefore, their incorporation into acylglycerols or wax esters may provide a defence strategy to regulate the fatty acid composition of the membrane phospholipids. Recently, Dahlqvist *et al.* (2000) identified an acyl-CoA-independent enzyme in plants, which is responsible for the biosynthesis of TAGs using phospholipids as acyl donor. This enzyme probably contributes only slightly to the accumulation of TAGs, but its role may be important for maintaining the functional fluidity of cellular membranes. If a similar enzyme occurs in strain PD630, it may be responsible for the biosynthesis of TAGs containing aromatic fatty acids. Assimilation of phenyldecane by strain PD630 was not complete under the cultivation conditions used in this study, since intermediates of phenyldecane oxidation were accumulated intracellularly, and others were excreted into the medium. These surplus intermediates may be further oxidized when an external carbon source becomes limiting.

In conclusion, this study provides a physiological and biochemical approach for investigating the catabolism and assimilation of phenyldecane by *R. opacus* strain PD630 under conditions of restricted growth. Such conditions normally predominate in natural environments. The understanding of the metabolic response of bacteria to the substances investigated in this study could be important not only for bioremediation processes but also for obtaining novel compounds, such as TAGs and wax esters containing aromatic compounds, by using biotechnological processes.

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