***Rhodococcus opacus* strain PD630 as a new source of high-value single-cell oil? Isolation and characterization of triacylglycerols and other storage lipids**

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The triacylglycerol (TAG)-accumulating, hydrocarbon-degrading bacterium *Rhodococcus opacus* strain PD630 and chemically induced storage-deficient mutants derived from this strain were investigated for their capability to accumulate storage lipids in the cytoplasm during cultivation under nitrogen-limiting conditions. Acylglycerols were analysed by matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) and by reversed-phase HPLC. Fatty acids comprising 13–19 carbon atoms in various acylglycerols constituted up to 76% of the cellular dry weight in gluconate-grown cells, with a significant proportion of odd-numbered fatty acids. Hydrolysis using pancreatic lipase and deacylation with ethyl magnesium bromide were employed to identify the stereospecific distribution of fatty acids at the glycerol. This analysis showed that the fatty acids were not randomly distributed between the three positions of the glycerol backbone. In comparison with common plant fats, where the longer and higher unsaturated fatty acids are predominantly found at position 2, *R. opacus* PD630 accumulated only the shorter and saturated fatty acids in this position. More than 100 mutants accumulating TAG at a significantly lower rate were obtained by chemical mutagenesis and identified by staining with Sudan Black B. All the mutants showed similar neutral lipid patterns by TLC analysis, with a small distinct spot exhibiting the same R_f value as TAG; this was identified as a residual amount of TAG by preparative TLC and MALDI-TOF, indicating that this bacterium is possibly capable of synthesizing TAGs by at least two different pathways.

Keywords: *Rhodococcus*, triacylglycerols, storage lipids

INTRODUCTION

Yeasts and moulds were recognized over a century ago to accumulate lipids (Ratledge, 1992). Since then, other lipid-accumulating micro-organisms have been identified among algae and also a few bacteria (Ratledge, 1989). Lipophilic storage compounds represent a convenient form for storing energy because of their relative

compactness, being anhydrous, and their much higher calorific value as compared to proteins or carbohydrates (Murphy, 1993). Many bacteria are able to accumulate specialized lipids, such as poly(3-hydroxybutyric acid) or other polyhydroxyalkanoic acids (PHAs) (Steinbüchel, 1991; Steinbüchel & Valentin, 1995). The accumulation of triacylglycerols (TAGs) has only been described for a few bacteria belonging to the genera *Mycobacterium* (Barksdale & Kim, 1977), *Streptomyces* (Olukoshi & Packter, 1994; Packter & Olukoshi, 1995), *Acinetobacter* (Makula *et al.*, 1975; Scott & Finnerty, 1976), *Nocardia* and *Rhodococcus* (Alvarez *et al.*, 1997). TAGs occur in bacteria as insoluble inclusions sur-

Abbreviations: ELSD, evaporative light-scattering detector; MALDI-TOF, matrix-associated laser desorption ionization–time of flight mass spectrometry; PHA, polyhydroxyalkanoic acid; TAG, triacylglycerol.

rounded by a membrane, as reported for *Streptomyces coelicolor*, *S. lividans* (Packter & Olukoshi, 1995) and *Rhodococcus opacus* PD630 (Alvarez *et al.*, 1996).

Recently, aerobic, Gram-positive bacteria belonging to the genus *Rhodococcus* have attracted increased interest in the biochemical and genetical characterization of their metabolic capabilities, based on their ability to transform or degrade diverse classes of mainly hydrophobic substances (Finnerty, 1992; Warhurst & Fewson, 1994). *Rhodococcus* spp. are also able to synthesize and accumulate PHAs after cultivation on different carbon sources under nitrogen-limiting conditions. The accumulation of a copolymer consisting of 3-hydroxybutyrate and 3-hydroxyvalerate from unrelated carbon sources was reported for *Rhodococcus ruber* (Haywood *et al.*, 1991). Alvarez *et al.* (1996, 1997) reported on an oleaginous hydrocarbon-degrading *R. opacus* strain, PD630, which is able to accumulate up to 76% of acylglycerols when grown on different carbohydrate and non-carbohydrate carbon sources under nitrogen-limiting conditions but is unable to synthesize PHAs.

The present work is concerned with the analysis of the composition and stereospecific structures of storage acylglycerols from *R. opacus* PD630, which have not been studied in detail previously. Stereospecific analysis of TAGs has been performed for many years to discriminate between fatty acids at the *sn*-1, -3 and -2 positions of TAG (Brockerhoff, 1965). In addition, we subjected *R. opacus* PD630 to chemical mutagenesis and screened for mutants defective in the accumulation of storage lipids.

METHODS

Bacterial strains, media and growth conditions. *R. opacus* PD630 (DSMZ 44193; Alvarez *et al.*, 1996) and the mutant *R. opacus* ROM34 derived from this strain (Kalscheuer *et al.*, 1999) were grown aerobically at 30 °C in 0.8% (w/v) nutrient broth medium or in mineral salts medium (MSM) according to Schlegel *et al.* (1961) with 1% (w/v) sodium gluconate as carbon source. The concentration of ammonium chloride was reduced to 0.01% (w/v) to promote the accumulation of TAGs. To obtain solidified media, 1.8% (w/v) agar was added.

Lipid extraction and TLC. Crude oil of *R. opacus* PD630 was obtained from lyophilized cell material (15 g), which was stirred twice each with 500 ml chloroform/methanol (2:1, v/v) at room temperature for 3 h. The solvents were evaporated from the combined extracts, and the residual material, 5.2 g, was dissolved in light petroleum (b.p. 30–40 °C) and applied to a column packed with 20 g silica gel 60 (mesh 50–200 µm, Merck) equilibrated with light petroleum. The column was eluted with 500 ml light petroleum; the total eluate was collected and evaporated to dryness to obtain 4.4 g of a yellowish crude oil fraction, which consisted of 90% fatty acids in total.

To determine the identity of the lipids, TLC was carried out on silica gel 60 F₂₅₄ plates (0.2 mm, Merck). For this, samples of lyophilized cell material were extracted with chloroform/methanol (2:1, v/v), and light petroleum (b.p. 30–40 °C)/

diethyl ether/acetic acid (70:30:1) was used as solvent. In some experiments, a double solvent system, benzene/acetic acid (85:15, v/v) followed by light petroleum (b.p. 30–40 °C) applied in the same direction, was used. Preparative TLC was carried out on silica gel plates of 2 mm thickness (Merck). Plates impregnated with methanolic silver nitrate or boric acid (each 5%, w/v) were also used. Lipid fractions were visualized by heating the plates over a Bunsen flame after spraying with 40% sulfuric acid. Components on preparative plates were visualized after brief exposure to iodine vapour. Quinone compounds were detected on silica gel TLC plates by specific staining with a leukomethylene blue spray reagent (Krebs *et al.*, 1967). Quinones appeared immediately as dark blue spots on a faint blue background. Osmium tetroxide vapour was used to visualize unsaturated compounds. 2-Monopalmitoylglycerol, tripalmitoylglycerol, 1,2-dipalmitoylglycerol, 1,3-dipalmitoylglycerol, cetylpalmitate, 1,2-dipalmitoyl-3-myristoyl-*rac*-glycerol, hexadecanol, tetradecene and oleic acid obtained from Sigma were used as reference substances.

Analysis of fatty acids. To determine the fatty acid content of the cells and the composition of the lipids, these compounds were treated to yield fatty acid methyl esters (Brandt *et al.*, 1988; Timm *et al.*, 1990), which were analysed by GC with a Perkin Elmer model 8420 gas chromatograph equipped with a Permaphase PEG 25Mx capillary column (25 m × 0.32 mm) and a flame ionization detector. A 2 µl portion of the organic phase was analysed after split injection (split ratio, 1:40); helium (32 ml min⁻¹) was used as carrier gas. The temperatures of the injector and detector were 230 °C and 275 °C, respectively, and a temperature programme was used for the separation of methyl esters (120 °C for 5 min; temperature increase of 8 °C min⁻¹; 180 °C for 37 min) on the column. The fatty acids were identified by comparison of their retention times with those of standard fatty acid methyl esters.

Reversed-phase HPLC. Reversed-phase HPLC was performed on an RP-18 Merck LiChroSphere 100 column (5 µm, 250 × 4.6 mm i.d.) using a Kontron HPLC apparatus equipped with a series 522 chromatographic pump. Elution of the compounds was monitored at 210 nm using a Kontron DAD 540 diode array detector (DAD) or an Alltech ELSD 500 evaporative light-scattering detector (ELSD) connected to a KromaSystem 2000 integrator system. The ELSD temperature was set to 55 °C. Acetonitrile/acetone (38:62, v/v) at a flow rate of 1 ml min⁻¹ was used as solvent system. To characterize TAG mixtures further, a partition number (PN) variable was used: $PN = CN - 2ND$, where CN and ND represent the total carbon number and the total number of double bonds in the fatty acids in the TAG molecule, respectively (Ruiz-Gutiérrez & Barron, 1995).

Matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI-TOF). MALDI-TOF was carried out using a LAZARUS III DE time of flight mass spectrometer (constructed by H. Luftmann, Institut für Organische Chemie, Münster, Germany) operated at 19 kV with delayed extraction and a path length of 2 m. A nitrogen laser was used to generate the primary beam, at 337 nm, with 3 ns pulse width. Samples were applied to the stainless steel target as 10⁻⁴ to 10⁻⁵ solutions (1 µl) mixed with an equal volume of a 0.1 M solution of 2,5-dihydroxybenzoic acid. The applied drop was allowed to dry and to crystallize before the sample was introduced into the mass spectrometer ion source.

Stereospecific analysis of TAGs. The procedure for the stereospecific analysis of TAGs is reported in the literature (Brockerhoff, 1965; Yurkowski & Brockerhoff, 1966; Christie

& Moore, 1968) and was applied to *R. opacus* PD630 crude oil as follows.

(i) Enzymic hydrolysis of TAGs was done with porcine pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3; Merck) to obtain *sn*-2-monoacylglycerols.

(ii) Partial deacylation of TAGs was done by chemical hydrolysis with ethyl magnesium bromide (Alfa Aesar).

(iii) Separation of enantiomeric *sn*-1,2(2,3)-diacylglycerols was achieved by TLC on boric-acid-impregnated silica gel plates, followed by treatment with phosphatidyl phenol (Sigma) to obtain synthetic L- and D-phosphatidyl phenols, which were subsequently hydrolysed by stereospecific phospholipase A₂ (EC 3.1.1.4) from rattlesnake venom (*Crotalus atrox*) to obtain the lysophosphatidyl phenols (Brockerhoff, 1965).

(iv) GC analysis of the fatty acid composition of TAGs, *sn*-2-monoacylglycerols and the lysophosphatidyl phenols was done as described above.

Mutagenesis. To obtain mutants deficient in TAG accumulation, *R. opacus* PD630 was subjected to chemical mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMG). Cells were incubated with 20 µg NMG ml⁻¹ for 30 min (50 % lethal rate). For expression of the mutations, the mutagenized cells were washed and resuspended in nitrogen-limited MSM containing 1 % (w/v) sodium gluconate. Enrichment of mutants was performed by Percoll density-gradient ultracentrifugation (Guerrero *et al.*, 1984). Since the buoyant density of cells of TAG-deficient mutants was higher than that of the wild-type cells, two distinct bands were obtained. The band representing the mutant cells was withdrawn from the gradient, plated on nitrogen-limited MSM agar plates containing 1 % (w/v) sodium gluconate and identified by staining with ethanolic Sudan Black B and subsequent destaining with ethanol (Schlegel *et al.*, 1970).

RESULTS

TLC pattern of neutral lipids of *R. opacus* PD630

The lipids from the crude organic extract were separated by TLC, isolated from the plates, and the structures of neutral storage lipids were elucidated by spectrometric methods. The TAG fraction accounted for about 90 % (w/w) of the total extractable lipids of *R. opacus* PD630. The spectrometric data obtained were compared with published data and with data from commercially available standards, resulting in the identification of TAG, 1,2-diacylglycerol and 1,3-diacylglycerol (see Fig. 2). A thin-layer chromatogram of the crude organic extract and the determined structures is shown in Fig. 1. The pattern of neutral lipids separated on TLC plates also included some compounds not representing storage lipids because of their relatively low concentration found in the extractable lipids, and some signals originated from the solvents and artefacts of extraction (referred to as 'unknown'). The intensities of these signals corresponded to the solvent volume used for cell extraction or were referred to the solvent systems used for TLC. Quinones were detected by specific staining on TLC plates with leukomethylene blue and are indicated in Fig. 1.

Fig. 1. TLC analysis of the crude organic extracts obtained from *R. opacus* PD630 and the TAG-deficient mutant *R. opacus* ROM70. The cells were grown in nitrogen-limited MSM containing 1 % (w/v) sodium gluconate for 48 h. Lipids were extracted and separated by TLC on silica gel plates as described in Methods. Solvent system: benzene/acetic acid (85:15, v/v) and then light petroleum (b.p. 30–40 °C) in the same direction. Lanes: 1, Cetylpalmitate; 2, tetradecene; 3, 2-monopalmitoylglycerol; 4, 1,2-dipalmitoylglycerol; 5, 1,3-dipalmitoylglycerol; 6, tripalmitoylglycerol; 7, crude lipid extract of *R. opacus* PD630; 8, crude lipid extract of *R. opacus* ROM70. DAG, diacylglycerol; MAG, monoacylglycerol. *Spots may result from impurities in the extraction solvent.

Fatty acid profile of accumulated TAGs of *R. opacus* PD630

The profile of fatty acids found in TAGs purified from gluconate-grown cells of *R. opacus* PD630 corresponded well with the fatty acid composition obtained from the analysis of intact cells (Table 1) and was in good agreement with those reported previously (Alvarez *et al.*, 1996). The fatty acids ranged in chain length from 13 to 19 carbon atoms, with the major fatty acids being 16:0, 17:0, 17:1 and 18:1. The only monomethyl-branched fatty acid that could be detected in the purified TAGs was tuberculostearic acid, present at trace levels (< 0.2 %, w/w); otherwise no other iso- or anteiso-fatty acids were detectable, as revealed by GC analysis at the Deutsche Stammsammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Therefore, the odd-numbered fatty acids were almost exclusively straight-chain fatty acids. Dienic fatty acids were not

Table 1. Distribution of fatty acids in TAGs from *R. opacus* PD630

Cells were cultivated for 48 h in low-nitrogen medium with 1% (w/v) sodium gluconate at 30 °C. Abbreviations and symbols: ND, not detectable; tr, traces (< 0.5%); –, could not be calculated.

	Fatty acid (mol%)									
	C13:0	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C19:0
Crude extract	ND	5.2	6.3	25.9	9.5	12.6	15.4	3.1	22.0	ND
Purified TAG	0.8	4.3	6.3	25.7	9.5	12.3	15.4	3.5	22.0	tr
<i>sn</i> -2	ND	11.3	11.7	59.3	tr	17.6	ND	tr	tr	ND
<i>sn</i> -1	ND	12.0	3.4	18.4	7.1	12.0	16.2	6.0	23.7	ND
<i>sn</i> -3 (calculated)	–	–	3.8	–0.6	21.4	7.3	30.0	4.5	42.3	–

Fig. 2. MALDI-TOF spectra of acylglycerols of *R. opacus* PD630 obtained after preparative TLC. (a) TAG fraction; (b) 1,2-diacylglycerol fraction; (c) 1,3-diacylglycerol fraction. All assignments correspond to $[M + Na]^+$.

detected. The saturated fatty acid content was over 52%, and the content of odd-numbered fatty acids was about 35%. Octadecenoic acid was identified as oleate (Δ^9 -octadecenoic acid) and was the major monoenic fatty acid.

Mass distribution of acylglycerols accumulated by *R. opacus* PD630

Following preparative TLC of acylglycerols, MALDI-TOF mass spectral data were acquired in order to obtain the molecular masses of the acylglycerols. MS data for

Fig. 3. HPLC separation of purified TAGs of *R. opacus* PD630 using an RP-18 Merck LiChroSphere column (5 μ m, 250 mm length $\times$ 4.6 mm inner diameter). Column temperature, 30 °C; mobile phase, acetonitrile/acetone (38:62, v/v) isocratic; flow rate, 1 ml min⁻¹. (a) UV detection at 210 nm; (b) detection by ELSD.

the purified TAG fraction revealed pseudomolecular ions $[M + Na]^+$ corresponding to total carbon units from 51 to 57 (Fig. 2a). MALDI-TOF spectra of purified 1,2- and 1,3-diacylglycerols (Figs 2b and 2c, respectively) revealed pseudomolecular ions corresponding to total carbon units from 32 to 38, indicating a similar fatty acid distribution as in the purified TAGs.

Reversed-phase HPLC

The reversed-phase HPLC analysis of purified TAG was carried out using UV detection at 210 nm and an ELSD to detect and distinguish between saturated and un-

Fig. 4. Separation of TAGs from *R. opacus* PD630 on a silver-nitrate-impregnated silica gel TLC plate. (a) The TLC plate was not impregnated; (b, c) the TLC plate was impregnated with 5% AgNO_3 . Plates (a) and (b) were stained with 40% sulfuric acid in the heat, whereas plate (c) was stained with osmium tetroxide vapour to visualize only unsaturated compounds. Lanes: 1, tripalmitoylglycerol (100 μg); 2, purified TAGs (200 μg); 3, purified TAGs (100 μg). UUU, TAGs with three monoenoic fatty acid residues; UUS, TAGs with two monoenoic fatty acid residues; USS, TAGs with one monoenoic fatty acid residue; SSS, TAGs with only saturated fatty acid residues.

saturated TAGs. The chromatograms showed approximately 20 individual peaks with estimated *PN* values between 39 and 53; UV and ELSD detection gave similar peak patterns (Figs 3a and 3b, respectively). Signals corresponding to *PN* values between 49 and 51 represented the most abundant peaks.

Argentation TLC

Purified TAGs of *R. opacus* PD630 were applied to silver-nitrate-impregnated TLC plates. The method of argentation TLC is based on the ability of olefinic bonds to form π -complexes with silver ions, enabling the separation of substances with different degrees of unsaturation. Under these conditions saturated TAGs migrate virtually with the solvent front, followed by the unsaturated TAGs in the order of an increasing degree of unsaturation. Since *R. opacus* PD630 accumulates only saturated and monoenoic fatty acids, this led to four different spots on the argentation TLC plate, with two abundant spots for TAGs containing one and two monoenoic fatty acids (Fig. 4).

Stereospecific analysis of TAGs of *R. opacus* PD630

The results of the stereospecific analysis are also shown in Table 1; they revealed a rather asymmetric distribution of fatty acids at the glycerol. The shorter and

saturated fatty acids tend to prefer position 2 against positions 1 and 3. The unsaturated fatty acids are predominantly found at position 3. Unsaturated fatty acids and acids with more than 17 carbon atoms were not found in significant amounts at position 2. Only small amounts of saturated fatty acids were found at position 3; palmitic acid does not appear to occur at this position. Therefore, the TAGs from *R. opacus* PD630 were quite different from those of mammals and plants, where the unsaturated longer fatty acids are predominantly found at position 2.

Acylglycerol accumulation in mutants of *R. opacus* PD630

R. opacus PD630 was subjected to chemical mutagenesis using NMG and screened for mutants defective in the accumulation of storage lipids by staining colonies with Sudan Black B. By this method we obtained more than 100 mutants (referred to as ROM, for *Rhodococcus opacus* mutants) which all exhibited a significantly lower content of TAGs and 1,2-diacylglycerols in comparison with the wild-type. They all also lacked detectable 1,3-diacylglycerols. In contrast, the amounts of monoacylglycerols were slightly higher as revealed by TLC analysis (see ROM70 in Fig. 1 as an example). A recorded MALDI-TOF spectrum with crude lipid extracts of mutant ROM34 did not indicate accumulation

Fig. 5. MALDI-TOF spectra of lipid crude extracts of (a) *R. opacus* PD630 and (b) *R. opacus* ROM34.

of any TAGs (Fig. 5). To identify the structure of the compound found at the same R_F value as TAGs in the TLC analysis, it was purified by preparative TLC, and the concentrated sample was analysed by MALDI-TOF. This analysis showed that it consisted of a similar mixture of TAGs to the wild-type. For further characterization of the isolated mutants, the spectra of the fatty acids which were incorporated into the various accumulated lipids were also analysed by GC after extraction and methanolysis. All mutants possessed a similar GC profile (data not shown). The amounts of the accumulated fatty acids were drastically reduced in all mutants, to less than 1% as compared to the wild-type. Therefore, the occurrence of monoacylglycerols in the mutants did not compensate for the lower TAG content. It is therefore obvious that all mutants were still able to synthesize and accumulate trace amounts of TAGs.

Investigation of wax ester accumulation

TLC analysis of *R. opacus* PD630 crude extracts (Fig. 1) revealed a faint signal with the same R_F value as cetylpalmitate; this signal was formerly referred to wax ester accumulation (Alvarez *et al.*, 1996). The compound was purified by preparative TLC and subjected to MALDI-TOF and electron-impact mass spectrometry (EI-MS) analysis, but data obtained by these methods did not confirm the presence of any wax ester (data not shown). Specific staining on TLC plates with leuko-methylene blue revealed a faint signal for this compound, indicating that it might contain a quinone structure. The recorded MALDI-TOF spectrum of this compound revealed two major signals differing in the length of two isoprene units with m/z values $[M + Na]^+$ of 550 and 685, which supports the presence of an additional quinone compound, but exact identification of these compounds could not be achieved.

DISCUSSION

R. opacus PD630 produced significant amounts of various acylglycerols that occurred as insoluble inclusions in the cells when the cells were cultivated on

sodium gluconate under nitrogen-limiting conditions. We demonstrated that the major fraction of the fatty acids accumulated by *R. opacus* PD630 are covalently linked to glycerol and occur as TAGs together with traces of diacylglycerols as revealed by TLC, GC and MALDI-TOF analysis. We showed that the pattern of lipophilic lipids seen in TLC analysis also contained some compounds which do not appear to function as storage lipids. Former indications of small amounts of wax esters (Alvarez *et al.*, 1997) could not be confirmed.

Stereospecific analysis of *R. opacus* PD630 TAGs to determine the distribution of fatty acids linked to either position of the glycerol backbone showed that fatty acids in the TAGs of *R. opacus* PD630 are not randomly distributed between the three possible positions. In contrast to common plant fats, where the longer and higher unsaturated fatty acids are found in position *sn*-2, *R. opacus* PD630 accumulated only the shorter and saturated fatty acids in this position. This indicates that the enzymes involved in TAG biosynthesis in *R. opacus* PD630 are highly specific and exhibit a substrate range different from the corresponding enzymes in plants.

In addition, we found that *R. opacus* PD630 synthesizes oleic acid ($18:1^{\Delta 9, cis}$), whereas vaccenic acid ($18:1^{\Delta 11, cis}$) was not detected. This provides evidence that *R. opacus* PD630 possesses the aerobic mechanism of fatty acid desaturation (Bloomfield & Bloch, 1960; Fulco *et al.*, 1964).

All mutants defective in TAG accumulation obtained in this study still accumulated very small amounts of TAG, which were less than approximately 1% of the wild-type. The results support the two following explanations. The remaining amounts of TAG could be explained by the existence of two different pathways for the biosynthesis of TAGs in *R. opacus* PD630. One pathway could be only slightly active and contribute only very small amounts of TAG in both the wild-type and the mutants. The second, major, pathway could be responsible for the accumulation of the major fraction of TAGs. If only one pathway existed, completely TAG-negative mutants should have occurred among the more than 100 mutants that were isolated and screened in this study. It is implied that all the mutants isolated were most likely defective in the major pathway; mutants impaired in the minor pathway were probably not detectable by the screening method applied, using the lipophilic dye Sudan Black B, due to the putatively only faint differences in the amounts of TAGs between the wild-type and this type of mutant that were expected. On the other hand, however, it is not excluded that all the mutants isolated were substantially impaired in the regulation of TAG production. This latter possibility could also lower the rate of TAG biosynthesis and give a similar mutant phenotype.

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