

Biosynthesis of fatty acids and triacylglycerols by 2,6,10,14-tetramethyl pentadecane-grown cells of *Nocardia globerula* 432

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Abstract

Nocardia globerula strain 432 was able to synthesize triacylglycerols (TAG) during cultivation on 2,6,10,14-tetramethyl pentadecane (pristane) under nitrogen-limiting conditions. Within these cells, 4,8,12-trimethyl tridecanoic acid was the major fatty acid detected. Fatty acids with an odd number of carbon atoms and minor amounts of even-numbered fatty acids were also observed. Experiments carried out with acrylic acid, an inhibitor of β -oxidation, suggested that odd-numbered fatty acids such as C15:0, C17:0 and 10-methyl C17:0 were synthesized de novo using propionyl-CoA, the β -oxidation product, as precursor. Although *N. globerula* 432 incorporated mainly straight chain fatty acids into TAG, the branched fatty acid 4,8,12-trimethyl tridecanoic acid also appeared, to some extent, in the acylglycerols. The importance of TAG biosynthesis by pristane-grown cells of *N. globerula* strain 432 is discussed. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Pristane; Fatty acid; Triacylglycerol; *Nocardia globerula*

1. Introduction

The genera *Rhodococcus* and *Nocardia* are nocardioform actinomycetes, Gram-positive bacteria, aerobic and widely distributed in nature. Recent studies demonstrated that these microorganisms accumulate triacylglycerols (TAG) during growth on different carbon sources, including hydrocarbons [1,2]. The accumulation of TAG in these bacteria greatly depended on the ammonium content in the culture medium [3]. The fatty acids occurring in TAG were directly related to the chain length of the substrate during cultivation of the cells on *n*-alkanes [1,2]. In addition, a bacterial strain identified as *Rhodococcus opacus* PD630 was able to accumulate into cellular inclusions some metabolites derived from the oxidation of the aromatic hydrocarbon phenyldecane, as well as neutral lipids,

when cells were cultivated under N-starvation [1]. The composition of the phenyldecane-derived intermediates indicated that the hydrocarbon was metabolized by β -oxidation and, to some extent, α -oxidation of the aliphatic side chain. The conversion of hydrocarbons into storage lipids during N-starvation is an interesting feature of these bacteria since these conditions are usually found in soil environments [4]. Moreover, these microorganisms have a broad metabolic spectrum for the transformation and degradation of different kinds of substrates, including a variety of alkanes, halogenated aliphatics and aromatics and other xenobiotic pollutants [5,6]. Several reports considered the potential of these bacteria for in situ bioremediation of the contaminated environment [4,7–9].

Highly branched isoprenoid alkanes, such as 2,6,10,14-tetramethyl pentadecane (pristane), normally occur in crude oil [10]. This hydrocarbon is more recalcitrant to biodegradation than other hydrocarbons. Methyl branching can block the β -oxidation pathway increasing the resistance of the hydrocarbon to biodegradation [10,11]. For this reason, the branched hydrocarbon pristane was used

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in some works as an internal standard in environmental hydrocarbon biodegradation processes [11,12]. However, pristane oxidation has been reported for different bacteria, such as *Brevibacterium* [13], *Corynebacterium* [14], and *Rhodococcus* [15].

In this study, we analyzed the response of the indigenous *Nocardia globerula* strain 432 to unbalanced growth conditions during cultivation on the recalcitrant branched hydrocarbon pristane. The ability of *N. globerula* strain 432 to use pristane for TAG biosynthesis in the absence of a nitrogen source in the medium allowed us to study not only the catabolism of the tetramethyl branched-chain alkane in this strain, but also the destination of the respective oxidation-derived intermediates.

2. Materials and methods

2.1. Bacterial strain, media and growth conditions

The isolation and characterization of *N. globerula* strain 432 were done in a previous study [4]. The strain was isolated from a soil sample collected in east Patagonia (Argentina). Cells were grown aerobically at 25°C in nutrient broth medium (0.8%, w/v) or in mineral salts medium (MSM) with the respective carbon source, according to Schlegel et al. [16]. To allow lipid accumulation, the concentration of ammonium chloride in the mineral medium was reduced to 0.05 g l⁻¹. To obtain solidified media, 1.8% (w/v) agar was added. Pristane was from Sigma, St. Louis, MO, USA.

2.2. Experiment with acrylic acid

Acrylic acid (Merck, Darmstadt, Germany) was used for the inhibition of fatty acid β -oxidation. Cells were grown aerobically at 25°C for 24 h in 500-ml flasks with 100 ml nutrient broth medium (0.8%, w/v) on a rotary shaker. After growth, the cells were harvested and resuspended in nitrogen-free medium containing 0.2% (v/v) pristane and 0.6 mg ml⁻¹ acrylic acid, incubated at 25°C for 24 h, harvested and analyzed for their chemical composition by gas chromatography–mass spectrometry (GC–MS) as described below.

2.3. Lipid extraction and thin layer chromatography (TLC)

In order to determine the identity of the lipids, TLC was carried out with samples of whole cells extracted with a mixture of chloroform:methanol (2:1, v/v). Chromatography was performed on 60F254 silica gel plates (Merck, Darmstadt, Germany) applying hexane–diethyl ether–acetic acid (80:20:1, v/v/v) as solvent system. Lipid fractions were visualized after brief exposure to iodine vapor. The following reference substances were used: palmitic acid,

stearic acid, dipalmitin, tripalmitin and cetylpalmitate (Merck, Darmstadt, Germany).

2.4. Analysis of fatty acids

For the determination of the fatty acid composition of lipids, 3–5 mg lyophilized whole cells, or the TAG fraction separated by preparative TLC were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid, and acyl- or hydroxyacyl-methylesters [1,17] were analyzed by GC coupled to a mass spectrometer TRIO 2, in a capillary column (50 m, 0.32 I.D.) with helium as gas carrier at 0.7 psi, temperature program: 150°C for 1 min, temperature increase 5°C min⁻¹, and 240°C for 10 min.

3. Results

3.1. Fatty acid composition of acetate- and pristane-grown cells



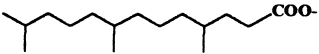

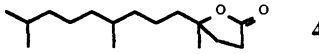

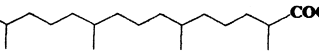
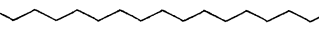
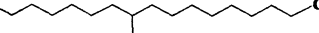


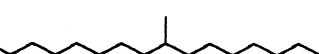
Cells of *N. globerula* 432 were cultivated in MSM with pristane as the sole carbon source, containing a reduced amount of ammonium. Under these conditions, cellular growth was restricted after ammonium exhaustion and they used the surplus carbon source mainly for TAG biosynthesis. After 4 days of incubation, cells were analyzed for their fatty acid composition. Table 1 shows the compounds detected by GC–MS analysis in pristane-grown cells of *N. globerula* 432 and their respective retention times.

Three classes of fatty acids were detected: (i) branched fatty acids, which derived directly from the carbon skeleton of pristane (fatty acids 3-5-7); (ii) even- and odd-numbered straight chain saturated and unsaturated fatty acids (fatty acids 1-2-4-6-8-10-11); and (iii) monomethyl branched fatty acids (compounds 9–12).

The fatty acid composition of cells cultivated on acetate under N-limiting conditions was used as reference for the analysis of fatty acid accumulation in pristane-grown cells (Table 1 and Fig. 1A). Fatty acids with an even number of carbon atoms, such as C16:0, C18:0, C18:1 and 10-methyl C18:0, were predominantly accumulated during cellular growth on acetate. Non-odd-numbered fatty acids were observed in these cells. The strain changed its fatty acid composition when grown on pristane as the only source of carbon and energy when compared to cells grown on acetate. The fraction of straight chain fatty acids in pristane-grown cells consisted mainly of odd-numbered fatty acids, such as C15:0 and C17:0, whereas the relative amounts of C16:0 and C18:0 decreased (Fig. 1A,B). C13:0 and C14:0 appeared only as traces in the cells. The only monomethyl branched fatty acid that could be detected in acetate-grown cells was tuberculostearic acid (compound 12). Although this one was also present in pristane-grown cells, a branched fatty acid with 17 carbon atoms (compound 9)

Table 1

Fatty acids and TAG synthesis by *N. globerula* 432 cultivated on 2,6,10,14-tetramethyl pentadecane and acetate

Fatty acids from cells grown on pristane					Fatty acids in	
R_f	Compounds		TAG	acetate-		
(min)			fraction	grown		
		(%)	(%)	cells (%)		
9.73	1 	C13:0	0.89	1.29	-	
11.26	2 	C14:0	0.34	2.98	1.95	
12.33	3 	4, 8, 12 trimethyl C13:0	35.10	11.35	-	
13.28	4 	C15:0	12.77	1.48	-	
15.00	5 	4, 8, 12 trimethyl C13:0-4-olide	9.39	-	-	
15.22	6 	C16:0	9.39	29.11	36.60	
16.52	7 	2, 6, 10, 14 tetramethyl C15:0	11.64	-	-	
17.15	8 	C17:0	5.90	1.98	-	
17.93	9 	10-methyl C17:0	6.49	-	-	
18.58	10 	C18:1	2.04	2.11	3.65	
19.02	11 	C18:0	0.69	49.70	33.25	
19.78	12 	10-methyl C18:0	5.36	-	24.55	

Cells were cultivated for 4 days in MSM containing 0.05 g l^{-1} ammonium chloride and pristane (0.2%, v/v) or sodium acetate (0.3%, w/v) as sole carbon sources. Triacylglycerols from pristane-grown cells were purified by preparative TLC as described in Section 2. Abbreviations: R_f , retention time; TAG, triacylglycerols; -, not detected.

was the major monomethyl branched fatty acid in these cells (Fig. 1A,B).

The only pristane oxidation products detected in the cells were 4,8,12-trimethyl tridecanoic acid (compound 3), as major product, and 2,6,10,14-tetramethyl pentadecanoic acid (compound 7) (Fig. 1B). They were consistent with a monoterminial oxidation of the branched hydrocarbon followed by β -oxidation of the pristanoic acid (compound 7). The occurrence of compound 5 in cells grown

with pristane suggested the formation of 4-hydroxy-4,8,12-trimethyl tridecanoic acid, which can lactonize to 4,8,12-trimethyl tridecan-4-olide as described by Rontani et al. [18].

3.2. Effect of acrylic acid on the fatty acid composition of cells grown on pristane

Cells of *N. globerula* 432 were cultivated in MSM under

N-starvation on pristane in the presence of 0.6 mg ml^{-1} of acrylic acid. This is an inhibitor of the β -ketothiolase of the β -oxidation pathway [19].

Cellular fatty acid composition changed in comparison to cells grown on pristane without the addition of the β -oxidation inhibitor to the medium (Fig. 1B,C). The most dramatic change was the increase in the relative amount of 2,6,10,14-tetramethyl C15:0 (compound 7) at the expense of 4,8,12-trimethyl C13:0 (compound 3), which is the first product of the β -oxidation of compound 7. In addition, fatty acids C15:0, C17:0 and 10-methyl C17:0 were practically absent in pristane-grown cells in the presence of acrylic acid. Products C16:0 (compound 6) and 10-methyl C18:0 (compound 12) were probably not synthesized by the cells during cultivation on pristane in the presence of acrylic acid but they may appear in the preculture incubation (Fig. 1C). These results suggested that straight chain and monomethyl branched fatty acids with an odd number of carbon atoms were synthesized *de novo* from precursors such as propionyl-CoA, which is an β -oxidation product of the pristanoic acid (compound 7).

3.3. Fatty acid composition of TAG accumulated by pristane-grown cells

Strain 432 was able to accumulate TAG during cultivation on pristane under N-limiting conditions. These neutral lipids were detected by TLC analysis of lipids extracted from intact cells, and were identified through the comparison of their R_f with those of reference compounds (R_f 0.81). TAG fraction was purified by preparative TLC and analyzed for its fatty acid composition by GC-MS (Table 1 and Fig. 1D). The major fatty acids present in TAG of pristane-grown cells were C16:0 (compound 6),

C18:0 (compound 11) and 4,8,12-trimethyl C13:0 (compound 13). However, the incorporation of this latter branched fatty acid in TAG was not proportional to its high relative content in cells during growth on pristane (Fig. 1B,D). The odd-numbered fatty acids that could be detected in purified TAG were present at trace levels; otherwise no monomethyl branched (10-methyl) fatty acids were detectable (Fig. 1D).

4. Discussion

Cells of *N. globerula* strain 432 were able to grow on the recalcitrant branched alkane pristane as the sole carbon and energy source. The results obtained in this work demonstrated that the strain studied continued using the hydrocarbon still under unbalanced growth conditions, and that the carbon source was mainly used for the biosynthesis and accumulation of TAG.

The cellular fatty acid composition of strain 432 depended on the compound used as carbon source as has already been reported for other TAG-accumulating bacteria [1,2]. The substrate used as carbon source may determine a pool of products available in the cells for the *de novo* fatty acid biosynthesis. During growth on acetate, it was evident that acetyl-CoA was the main precursor for fatty acid biosynthesis; therefore, fatty acids with an even number of carbon atoms predominated in the cells. In contrast, the fatty acid composition of pristane-grown cells depended on the oxidation reactions of the hydrocarbon. Results suggested that pristane was degraded by monoterminial oxidation to the corresponding carboxylic acid, pristanoic acid, and then, this product was β -oxidized to 4,8,12-trimethyl tridecanoic acid (Fig. 2). These oxidation

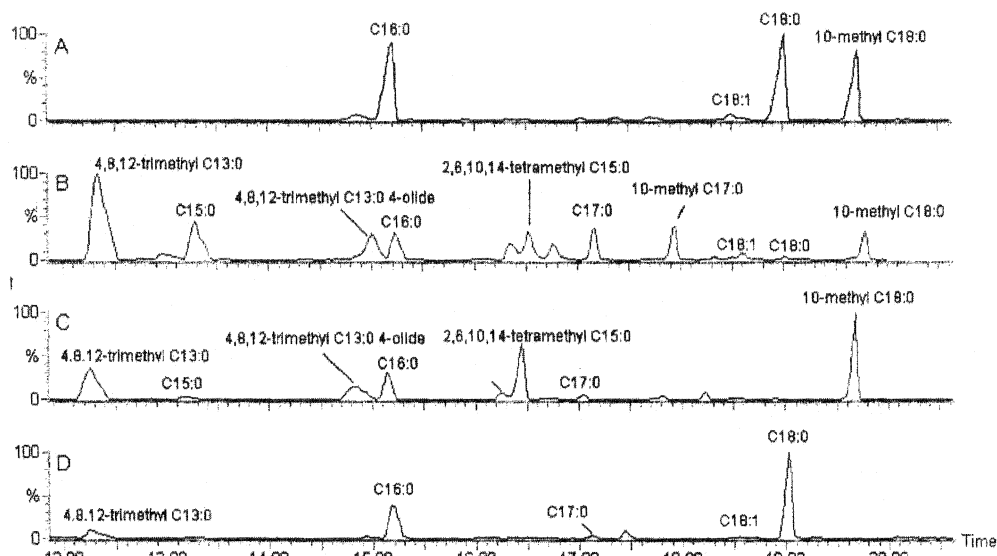


Fig. 1. Fatty acids from cells of *N. globerula* 432 grown on acetate or pristane and from TAG accumulated by pristane-grown cells. A: GC of fatty acids ME from acetate-grown cells; B: GC of fatty acids ME from pristane-grown cells; C: GC of fatty acids ME from cells cultivated on pristane in the presence of acrylic acid; D: GC of fatty acids ME from TAG purified from pristane-grown cells.

pathways have also been reported for *Brevibacterium* sp. [13], *Corynebacterium* sp. [14] and *Rhodococcus* sp. [15]. Previous studies also reported the occurrence of ω -oxidation in pristane-grown cells, but we did not detect ω -oxidation products in strain 432.

The major compound in cells cultivated on pristane under N-starvation conditions was 4,8,12-trimethyl tridecanoic acid. β -Oxidation was probably not complete to 2-methyl propionyl-CoA derivative under the conditions applied in this study, since no more branched fatty acids with a skeleton of two carbon atoms shorter than C13:0 were detected. In addition, no iso branched-chain fatty acids, which would probably be synthesized de novo from 2-methyl propionyl-CoA as precursor, occurred in the cells. Similar results had previously been reported for *Rhodococcus* bacteria during cultivation on *n*-alkanes under nitrogen-limiting conditions [2]. In these cells, precursors acetyl-CoA and propionyl-CoA were not available for the biosynthesis of polyhydroxyalkanoates and fatty acids during cultivation on the mentioned hydrocarbons.

Cultivation experiments performed in the presence of acrylic acid suggested that the β -oxidation pathway was the main source of propionyl-CoA, which was the compound used as precursor for the de novo biosynthesis of fatty acids with an odd number of carbon atoms (Fig. 2). This may account for the occurrence of odd-numbered fatty acids as major compounds in cells cultivated on pristane, in contrast to those grown on acetate. Among these fatty acids, it is worth mentioning the formation of 10-methyl heptadecanoic acid in pristane-grown cells of strain 432. Monomethyl branched fatty acids, mainly 10-methylstearic acid (tuberculostearic acid), are characteristic components of Gram-positive bacteria belonging to the genera *Mycobacterium*, *Rhodococcus*, *Nocardia* and *Streptomyces* [20,21]. 10-Methyl fatty acids are normally formed from

the addition of the *S*-methyl group of *S*-adenosyl-L-methionine to an unsaturated fatty acid residue of a phospholipid [20]. These branched fatty acids were not detected in TAG accumulated by pristane-grown cells of *N. globerula* 432, suggesting that they were likely to be incorporated into cellular membranes (Fig. 2). This was in agreement with the study of Wältermann et al. [22], who reported the occurrence of tuberculostearic acid only at trace levels in TAG accumulated by *R. opacus* PD630.

Our results suggested that the fatty acid composition of TAG synthesized by *N. globerula* 432 was determined by the specificity of the acyltransferase enzymes involved in the biosynthesis of these acylglycerols. Mainly straight even-numbered fatty acids (C16:0 and C18:0) were incorporated into TAG. In this context, normal fatty acids (C16:0, C17:0, C17:1 and C18:1) were also channeled preferably into TAG by gluconate-grown cells of *R. opacus* PD630 as revealed in a previous study [22]. Probably because of its high availability in the cells, 4,8,12-trimethyl tridecanoic acid was the only branched fatty acid esterified in the acylglycerols by strain 432 (Fig. 2). This indicated that the enzymes involved in TAG biosynthesis of strain 432 recognize the trimethyl branched fatty acid as substrate.

In conclusion, this study provides evidence that *N. globerula* 432 was able to degrade the recalcitrant branched hydrocarbon pristane still under growth restricting conditions, which normally predominate in the environment, and to produce cellular lipids from the oxidation products of the hydrocarbon. In contrast, under unbalanced growth conditions, many bacteria block lipid metabolism, such as the de novo fatty acid biosynthesis and β -oxidation pathways, through the limitation of essential nutrients [23]. This property makes this indigenous strain a promising candidate for bioremediation purposes in the

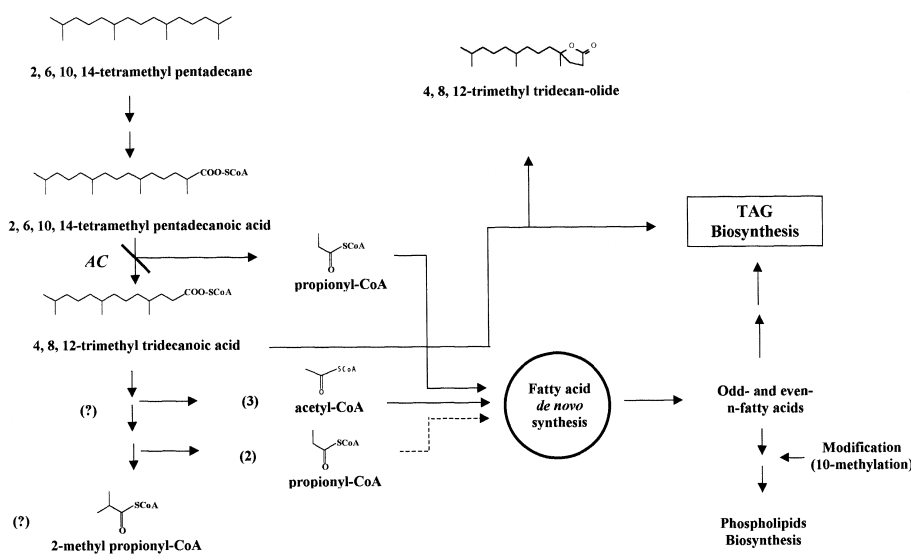


Fig. 2. Pathways for fatty acid and TAG biosynthesis proposed for *N. globerula* 432 during culture on pristane as the sole carbon and energy source under nitrogen-limiting conditions. Oxidation reactions involved in pristane catabolism on the left side seem to be not complete to 2-methyl propionyl-CoA under the conditions used in this study. Abbreviations: TAG, triacylglycerols; AC, acrylic acid.

environment. The understanding of the response of these bacteria to polluting substances and to environmental factors is of great importance for predicting and manipulating bacterial activities in the environment.

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