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Characterization of indigenous *Rhodococcus* sp. 602, a strain able to accumulate triacylglycerides from naphthyl compounds under nitrogen-starved conditions

Roxana A. Silva [a], Vincent Grossi [b], Nelda L. Olivera [c], Héctor M. Alvarez [a,*]

[a] *Centro Regional de Investigación y Desarrollo Científico-Tecnológico (CRIDECIT), Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, Km 4 Ciudad Universitaria, 9000 Comodoro Rivadavia, Chubut, Argentina*

[b] *Université Lyon 1, CNRS, UMR5125, Paléoenvironnements et Paléobiosphère, Campus Scientifique de la Doua, F-69622 Villeurbanne, France*

[c] *Centro Nacional Patagónico (CENPAT – CONICET), Boulevard 2915, 9120 Puerto Madryn, Chubut, Argentina*

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Abstract

An indigenous bacterium (strain 602) isolated in this study from a polluted soil sample collected in Patagonia (Argentina) was investigated in relation to its metabolic responses under unbalanced growth conditions. This strain was identified as *Rhodococcus* sp. by molecular analyses. Strain 602 showed the ability to degrade a wide range of compounds and to synthesize triacylglycerols under nitrogen-limiting conditions. Cells were also able to accumulate triacylglycerols during cultivation on naphthalene and naphthyl-1-dodecanoate. Triacylglycerols produced by resting cells in the presence of naphthyl-1-dodecanoate contained only short-chain length fatty acids (from C_8 to C_{12}), suggesting an initial attack of the substrate by an esterase releasing 1-naphthol and dodecanoic acid, which was subsequently degraded by β-oxidation. On the other hand, naphthalene seemed to be degraded by a mono-oxygenase yielding 1-naphthol, which was then transformed to 4-hydroxy-1-tetralone and to other possible metabolic intermediates. On the basis of the results obtained, a pathway involved in the metabolism of both aromatic compounds under nitrogen starvation by strain 602 is proposed. The results also demonstrated that *Rhodococcus* sp. 602 maintains its metabolic activity even in the absence of a nitrogen source. Intracellular triacylglycerols may help cells to maintain their catabolic activities under these growth-restricting conditions.

Keywords: *Rhodococcus*; Triacylglycerols; Naphthalene; Naphthol; Naphthyl-1-dodecanoate; Nitrogen starvation

1. Introduction

Rhodococcus is a versatile genus able to biodegrade a broad range of environmental contaminants (Larkin et al., 2005; Martínková et al., 2009; Warhurst and Fewson, 1994). Rhodococci usually possess very large genomes that contain multiple catabolic enzymes and pathways, as revealed by the available genome databases. In addition, they frequently harbor several small circular and large linear plasmids that contribute to their metabolic versatility and biochemical diversity. In this context, geographic sites with extreme environmental conditions such as semi-arid Patagonia (Argentina) may be important sources of indigenous rhodococcal strains adapted to extreme conditions and possessing new physiological capabilities. Members of this genus possess the capacity for degrading organic compounds even under growth-restricting conditions, which predominate in natural environments such as soils. Under these conditions, cells transform hydrocarbons principally into lipids, such as triacylglycerols (TAG) or wax esters (WE), which are accumulated as intracellular inclusion bodies (Alvarez, 2006). For instance,

* Corresponding author. Tel./fax: +54 297 4550339.
E-mail addresses: rsilva@unpata.edu.ar (R.A. Silva), vincent.grossi@univ-lyon1.fr (V. Grossi), olivera@cenpat.edu.ar (N.L. Olivera), halvarez@unpata.edu.ar (H.M. Alvarez).

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Table 1
Oligonucleotides and PCR conditions used in this study.

Gene	Primer	Sequence (5′ → 3′)	PCR conditions
16 rDNA	27F	AGAGTTTGATCCTGGCTCAG	90 °C 5 min, −94 °C 45 seg, 58 °C 45 seg, 72 °C 45 seg − 30 times, 72 °C 10 min
	518R	ATTACCGCGGCTGCTGG	
	357F	CTCCTACGGGAGGCAGCAG	94 °C 5 min, −94 °C 1 min, 62 °C 45 seg, 72 °C 1 min − 35 times, 72 °C 7 min
	1087R	CTCGTTGCGGGACTTACCCC	
	945F	GGGCCCGCACAAGCGGTGG	94 °C 5 min, −94 °C 1 min, 55 °C 1 min, 72 °C 1 min − 35 times, 72 °C 10 min
	1492R	GGTTACCTTGTTACGACTT	
*gyr*B	RB2F	GAGATGGCCTTCCTCAAC	94 °C 10 min, −94 °C 1 min, 61 °C 1 min, 72 °C 2 min − 40 times, 72 °C 10 min
	RB6R	GTACTTGTTGACCGTCGCC	
ws/*dgat*	5′-primer	ATGACCCAGACGGACTTC	90 °C 5 min, −94 °C 30 seg, 55 °C 30 seg, 72 °C 1 min 30 s − 30 times, 72 °C 5 min
	3′-primer	TCACGAGGCCACGACCAC	

Rhodococcus opacus PD630 accumulated a mixture of TAG and WE containing phenyldecanoic acid residues when cultivated with phenyldecane as sole carbon source under nitrogen-limiting conditions (Alvarez et al., 2002). *Nocardia globerula* and *Mycobacterium ratisbonense*, which are taxonomically related to *Rhodococcus* genus, were able to accumulate TAG and WE containing isoprenoid fatty acids (such as 4, 8, 12-trimethyltridecanoic acid) and fatty alcohols, (such as 2, 6, 10, 14-tetramethylhexadecan-1-ol) after cultivation on pristane and phytane, respectively (Alvarez et al., 2001; Silva et al., 2007). The ability of rhodococci to degrade and transform a diversity of contaminants into lipids under growth-restricting conditions may be the result of the richness of catabolic and anabolic enzymes in their genomes. This is highlighted by the redundancy and the non-specificity of WE synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) enzymes involved in TAG and WE biosynthesis. Bacterial WS/DGAT's are non-specific enzymes that accept a broad range of compounds as substrates for in vivo and in vitro acylation (Kalscheuer et al., 2003; Stöveken et al., 2005; Uthoff et al., 2005). The ability of rhodococci to convert diverse hydrocarbons into lipids under nutritional stress may allow cells to maintain their catabolic activity under growth limiting conditions. These properties are of great importance for predicting microbial degradation (bioremediation) activities in nutritional-poor soil environments, such as in Patagonia. The crude oil industry is the main economic activity in this region. Hydrocarbon spills have occurred at many sites in the region, and there is a need to remediate contaminated soil. For these reasons, we were interested in the isolation of indigenous *Rhodococcus* members and related bacteria with the ability to degrade polyaromatic hydrocarbons (PAHs) and to produce storage lipids from these hydrocarbons. Naphthalene is a representative PAH widely distributed in the environment. The first step of its bacterial degradation generally involves a ring cleavage catalyzed by 1,2-dioxygenase enzymes, yielding 1,2-dihydro-1,2-dihydroxynaphthalene. This latter compound is then metabolized into salicylic acid and to cathecol (Bosch et al., 2000). However, alternative metabolic reactions or intermediates have been reported. Strains of *Ralstonia* and *Rhodococcus* released gentisate from salicylic acid instead of cathecol (Di Gennaro et al., 2001; Grund et al., 1992; Zhou et al., 2001). In *R. opacus* M213, naphthalene is not metabolized via salicylate (Uz et al., 2000), whereas, in a thermophilic bacterium, metabolites corresponding to 1,2- and 2,3-dioxygenases catalysis were detected simultaneously (Annweiler et al., 2000). Also, in several fungi, naphthalene is transformed mainly into 1-naphthol (67.9%) and, to lesser extent, into 4-hydroxy-1-tetralone (16.7%) via 1,4-naphthoquinone (Augustin et al., 2006; Cerniglia and Gibson, 1977). Similar catabolic pathways were reported for cyanobacteria and microalgae (Cerniglia et al., 1980), human cells (Waidyanatha et al., 2004) and only for two bacteria, *Bacillus cereus* and *Streptomyces griseus* NRRL8090 (Cerniglia et al., 1984; Gopishetty et al., 2007).

In the present study, a strain belonging to the *Rhodococcus* genus was isolated from a contaminated soil of Patagonia and investigated for its ability to convert naphthyl-derivatives into lipids under growth-restricting conditions. Indigenous hydrocarbon-degrading bacteria could be an interesting source of industrial and environmental relevant applications.

2. Materials and methods

2.1. Media, culture conditions and chemical compounds

Cells were grown aerobically at 28 °C in nutrient broth medium (NB) (0.8%, w/v) or in mineral salts medium (MSM) (Schlegel et al., 1961) in the absence (MSM0) or the presence of 0.1 or 1 g l^{-1} NH_4Cl (MSM0.1 or MSM1, respectively). Solidified medium was obtained by adding 1.4% (w/v) of agar–agar.

Chemical compounds were obtained from Sigma–Aldrich (St. Louis, MO), Merck (Darmstadt–Alemania or Spain) or Fluka–Chemika (Switzerland).

2.2. Isolation of indigenous bacterial strains

A soil sample (100 g) was randomly collected from a chronically hydrocarbon-contaminated area near the Comodoro Rivadavia (45°47′S/67°30′W), Patagonia, Argentina. Soil particles were distributed on the surface of an MSM1 agar plate with *n*-hexadecane as sole carbon and energy source. *n*-Hexadecane was not directly included in the solid medium but was provided on a filter paper disc in the lid of the Petri dish. Colonies which grew after 7 days of incubation at 28 °C were isolated on NB agar plates.

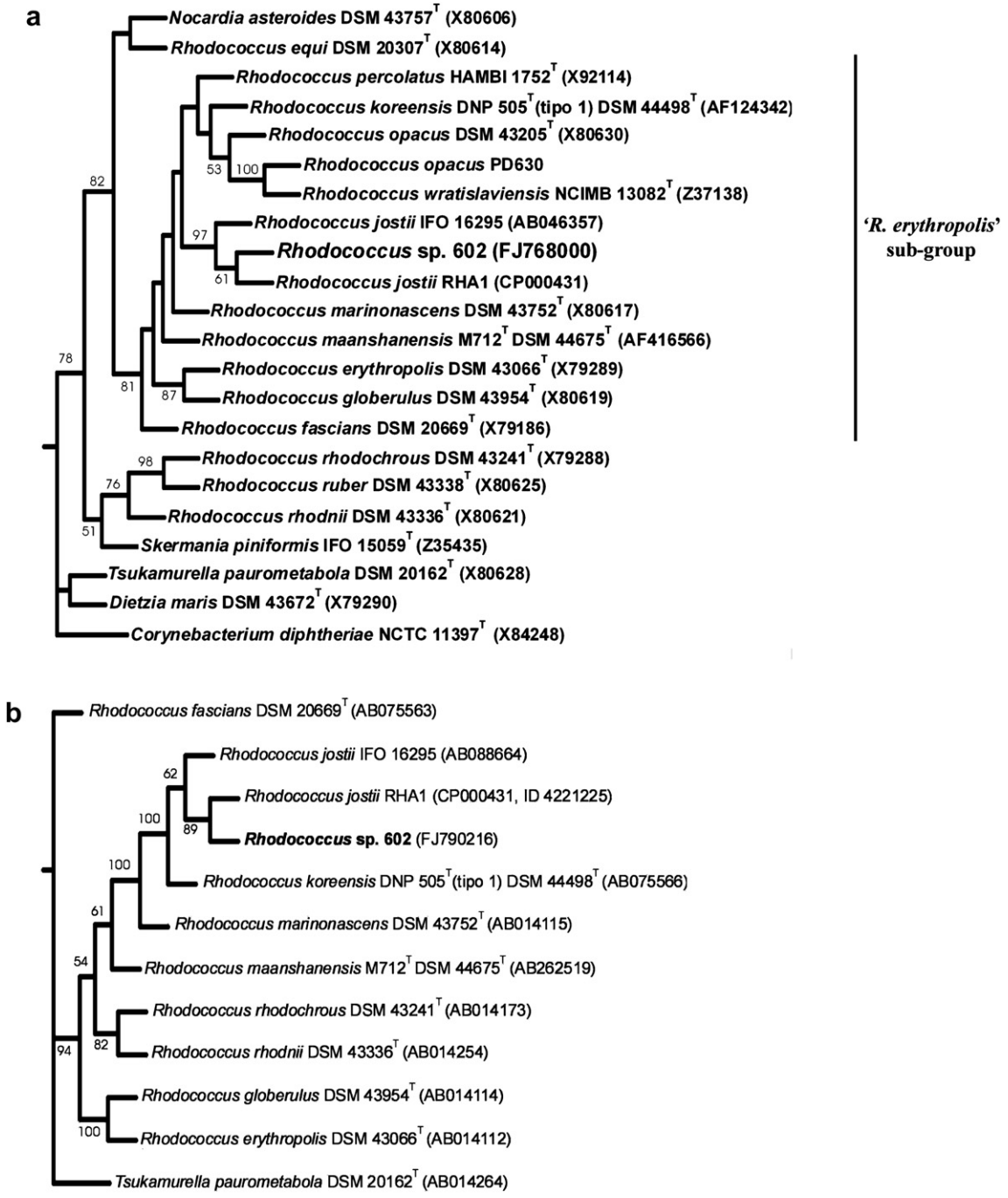


Fig. 1. Neighbor-joining trees based on (a) 16S rDNA (approximately 1400 bp) and (b) *gyr*B (approximately 400 bp) sequences showing relationships among strain 602 and representatives of the suborder *Corynebacterineae*, including the type strains of described species of the genus *Rhodococcus*. Numbers at internal nodes are bootstrap support values (%). GenBank accession numbers are given in parentheses.

A preliminary characterization of intracellular lipid accumulation was performed with Sudan Black B according to Schlegel et al. (1970). Strain 602 was selected among other indigenous isolates for its ability to accumulate large amounts of intracellular lipids.

2.3. Physiological characterization of strain 602

A preliminary physiological characterization of strain 602 was performed by determining the following criteria: cell shape, colony shape and color, Gram stain, catalase, oxidase and qualitative enzyme tests (DNase and urease). Tolerances to pH, temperature and NaCl concentration were determined on nutrient broth incubated for up to 7 days.

2.4. Taxonomic characterization and phylogenetic analysis

Cells were lysed and DNA was extracted using a Gen Elute™ NA2100 (Sigma) bacterial genomic kit. The 16S

Table 2
Fatty acid composition of *Rhodococcus* sp. strain 602 after cultivation on gluconate, benzoate and *n*-hexadecane under nitrogen-limiting conditions.

Carbon source	PHA (% CDW)	3HB (mol%)	3HV (mol%)	Fatty acids (% CDW)	Relative proportion of fatty acids (%, w/w)							
					$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$
Gluconate	9.0	16.7	83.3	71.2	1.7	3.1	30.2	7.8	10.9	8.9	17.5	19.9
Benzoate	8.2	25.0	75.0	64.9	3.4	6.2	30.7	11.3	10.3	11.3	11.9	14.9
n-Hexadecane	nd	nd	nd	22.3	16.3	nd	51.4	32.3	nd	nd	nd	nd

Abbreviations: CDW, cellular dry weight; nd, not detected; PHA, polyhydroxyalkanoates; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate.
Cells were cultivated for 48 h in liquid mineral medium with 0.05 g l^{-1} NH_4Cl and carbon sources added at 0.1–1% (v/v or w/v).

rRNA gene of this strain was amplified by PCR with universal primers for eubacteria (Table 1). *gyr*B and *ws/dgat* genes were amplified by PCR according to Taki et al. (2007) and Alvarez (2009), respectively (Table 1). In all cases, amplification was performed with a thermal cycler (Eppendorf Mastercycler Personal) using a 25 µl mixture containing 1–1.25 U of Taq DNA polymerase (InbioHighway, Argentina), 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 1.25% (v/v) DMSO, each deoxynucleotide triphosphate at a concentration of 200 µM, 25 pmol of each primer and 2 µl of appropriate concentrations of the extracted template DNA.

Sequencing was performed on both strands by the commercial services of Macrogen Inc. (Seoul, Korea). Sequences were screened against the NCBI database using the BLAST search program. For phylogenetic analyses, sequences were aligned using the T-Coffee method (Notredame et al., 2000) and were processed by the Genedoc program (Nicholas et al., 1997). Evolutionary trees were inferred using maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge and Farris, 1969) and neighbor-joining (Saitou and Nei, 1987) methods. *Corynebacterium diphtheriae* NCTC 11397^T 16S rRNA gene (GenBank Accession No. X84248) and *Tsukamurella paurometabola* DSM 20162^T *gyr*B gene (GenBank Accession No. AB014264) were used as outgroup in each case. The resultant tree topologies were evaluated by carrying out bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings, using the SEQBOOT, DNADIST and CONSENSE programs in the PHYLIP package (Felsenstein, 1993).

Table 3
Lipid composition (% of total lipids determined by TLC-FID) of cells of *Rhodococcus* sp. 602 grown during 6 days on naphthalene and naphthyl-1-dodecanoate in the absence of the nitrogen source.

Lipid class	Nah	$NahO_2C_{12}$
TAG	48.8	7.4
FA	6.6	68.2
ALC	12.4	ND
1,3DG	1.6	ND
1,2DG	1.4	ND
PE	1.9	4.5
MGDG	1.6	4.1
DGDG	10.7	7.3
DPG + PG	13.8	6.5
Others[a]	1.2	2

Abbreviations: Nah, naphthalene; $NahO_2C_{12}$, naphthyl-1-dodecanoate; TAG, triacylglycerides; FA, fatty acids; ALC, alcohols; 1,3DG, 1,3-diacylglycerols; 1,2DG, 1,2-diacylglycerols; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; DPG + PG, diphosphoglycerides + phosphoglycerides; PE, phosphatidylethanolamine.
[a] Uncharacterized colored background eluting at a place where pigments generally elute.

2.5. Determination of hydrocarbon utilization

The ability of strain 602 to utilize different compounds as sole carbon source was studied by visible cellular growth (formation of colonies) after 10 days at 28 °C on solid MSM1 medium. Substrates were added at different concentrations: glucose, lactose, sucrose and sodium valerate (0.5%, w/v); sodium acetate, sodium citrate, sodium octanoate, soluble extract of petroleum and squalane (0.2%, v/v); sodium benzoate (0.3%, w/v); sodium gluconate (1%, w/v); glycerol, naphthylethylenediamine dichlorohydrate and phytane (0.1%, v/v). Airplane gasoline, alkyl benzene sulfonic acid, benzene, bio-orange (a biodegradable detergent), butanol, chloro-benzene, 3-cyclohexylpropionic acid, diesel oil, *n*-ethanol, gasoline, *n*-hexane, *n*-hexadecane, naphthalene, phenol, 2-propanol, *n*-tetradecane, toluene and xylene were not directly included in the solid medium, but were provided on a filter paper disc in the lid of the Petri dish. Meanwhile, solid hydrocarbons and derivatives such as anthracene, fluorene, naphthyl-1-dodecanoate, naphthyl-1-hexadecanoate, octadecylamine and phenanthrene were dissolved in chloroform and distributed on the surface of agar plates and the solvent was evaporated.

Determining cellular growth by measuring the OD, dry weight, cell counts or protein content was very difficult when cells were cultivated with hydrocarbons due to the formation of large cellular flocs with low buoyant densities. For this reason, we measured mineralization of substrates as an indicator of cell activity. The time-dependent release of CO_2 by strain 602 associated with the utilization of different hydrocarbons as carbon sources was followed according to a modified method of Fredrickson et al. (1991). Cells were grown overnight at 28 °C in 10 ml NB medium on a rotary shaker. After growing, cells were harvested, washed once with sterile NaCl solution (0.85%, w/v) and resuspended in the same solution to obtain an OD_{436} of 5. Two milliliter aliquots were used to inoculate 250 ml flasks containing 40 ml MSM1 (balanced conditions) with 0.1% (w/v) of a single hydrocarbon (or derivative) as sole carbon source (anthracene, 3-cyclohexylpropionic acid, fluorene, glycerol, *n*-hexadecane, naphthalene, naphthyl-1-dodecanoate, octadecylamine, phenanthrene and sodium benzoate). Each flask was designed to receive a vial containing 2 ml of 1 M NaOH to absorb CO2 produced by

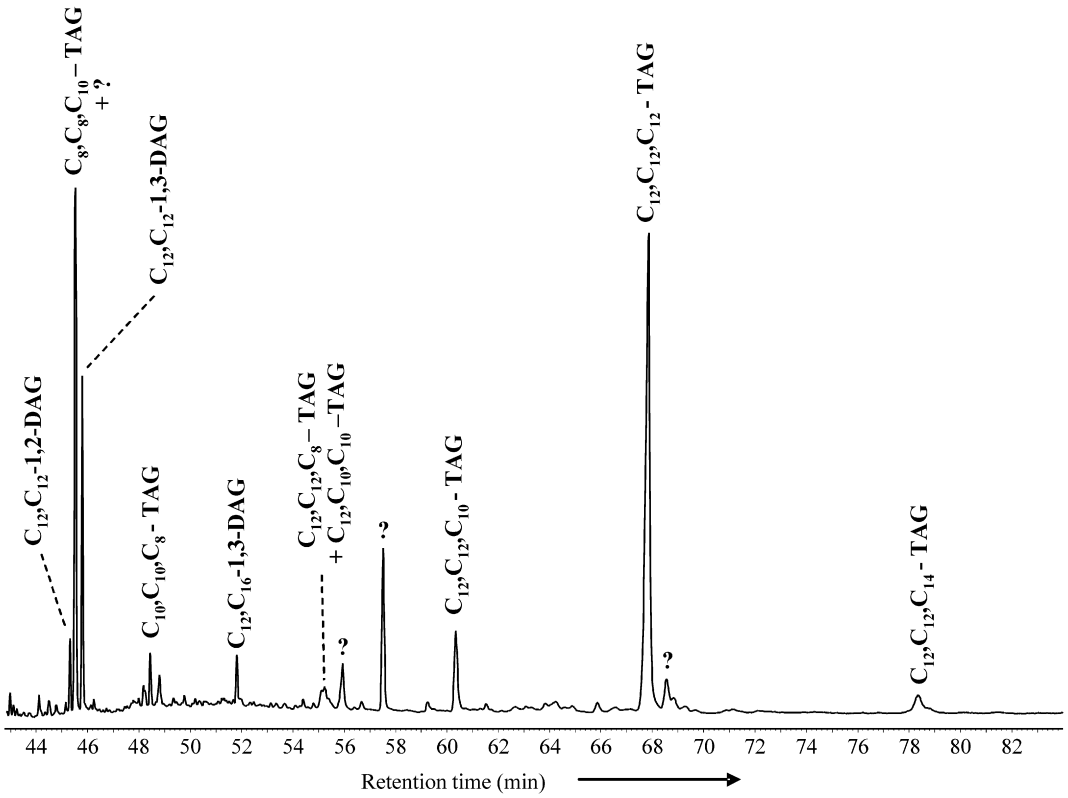


Fig. 2. Partial total ion chromatogram (TIC) of the silylated neutral lipids of *Rhodococcus* sp. strain 602 grown on naphthyl-1-dodecanoate under N-starved conditions. Reference: DAG, diacylglycerol; TAG, triacylglycerol; ?, unidentified compound.

the cells. These vials were removed every 24 h and replaced by new vials containing a fresh NaOH solution. The flasks were tightly sealed with wrapped rubber stoppers and incubated for 7 days at 28 °C on a rotary shaker. CO_2 production was monitored by titration with 0.1 M HCl.

2.6. Extraction and analysis of cellular lipids

Cells grown on NB were resuspended in MSM medium containing 0.05–0.1 g l^{-1} ammonium chloride (N-deficient or unbalanced growth conditions) and *n*-hexadecane (0.1%, v/v), sodium gluconate (1%, w/v) or benzoate (0.28%, w/v) as carbon source, and were incubated for 2 days in order to determine the storage lipid composition of cells from those carbon sources. Cells were harvested, washed twice with a sterile saline solution (0.85% NaCl, w/v) and lyophilized. Freeze-dried cells were extracted with methanol–chloroform (MeOH–$CHCl_3$, 1:2, v/v) and an aliquot of the total extract was separated by thin layer chromatography (TLC) on a 60F254 silica gel plate (Merck) applying *n*-hexane–diethyl ether–acetic acid (80:20:1, v/v/v) as a solvent system. Lipid fractions were revealed using iodine vapour. Tripalmitin and cetylpalmitate were used as standards.

The total fatty acid content of these cells was also determined by methanolysis (MeOH–H_2SO_4 15%, v/v) of lyophilized cells (5–10 mg). Fatty acid methylesters were analyzed using an HP5890A gas chromatograph equipped with an InnoWAX capillary column (30 m × 0.53 mm × 1 μm) and a flame ionization detector, using helium as carrier gas (13 ml min^{-1}). The temperatures of the injector and detector were 270 °C and 320 °C, respectively. The oven temperature was maintained at 90 °C for 5 min, then programmed at 6 °C by minute to 240 °C at which it was held for 17 min. For quantitative analyses, tridecanoic acid was used as an internal standard.

To investigate naphthyl-compound metabolism under N-starved conditions, cells grown on a rotary shaker for 24 h in three 250 ml flasks with 50 ml of NB were harvested, washed twice with a sterile saline solution (0.85% NaCl, w/v) and resuspended in MSM0 containing 0.1% (w/v) of naphthalene or naphthyl-1-dodecanoate (resting cells). After 6 days of incubation, cells were harvested, washed twice with the sterile saline solution, lyophilized and analyzed for their lipidic content. The different lipid classes were quantified by thin layer chromatography-flame ionization detection (TLC-FID), as described by Caradec et al. (2003). An aliquot of the total lipid extract obtained by MeOH–$CHCl_3$ extraction (1:2, v/v) was separated on chromarods and analyzed on a TLC-FID Iatroscan TH10 apparatus model MK-IV (Iatron, Japan). The separation scheme involved successive elution steps in solvent systems of increasing polarity. Lipid classes were quantified with an external calibration using a mixture of standard lipids.

Another aliquot of the total lipid extract was chromatographed over a wet packed (*n*-hexane) column of silica gel and three fractions were eluted with $CHCl_3$, acetone and MeOH yielding neutral compounds, glycolipids and phospholipids, respectively. All alcohols and non-esterified acids were silylated by reaction with bis-trimethylsilyl-trifluoroacetanamide (Supelco) in

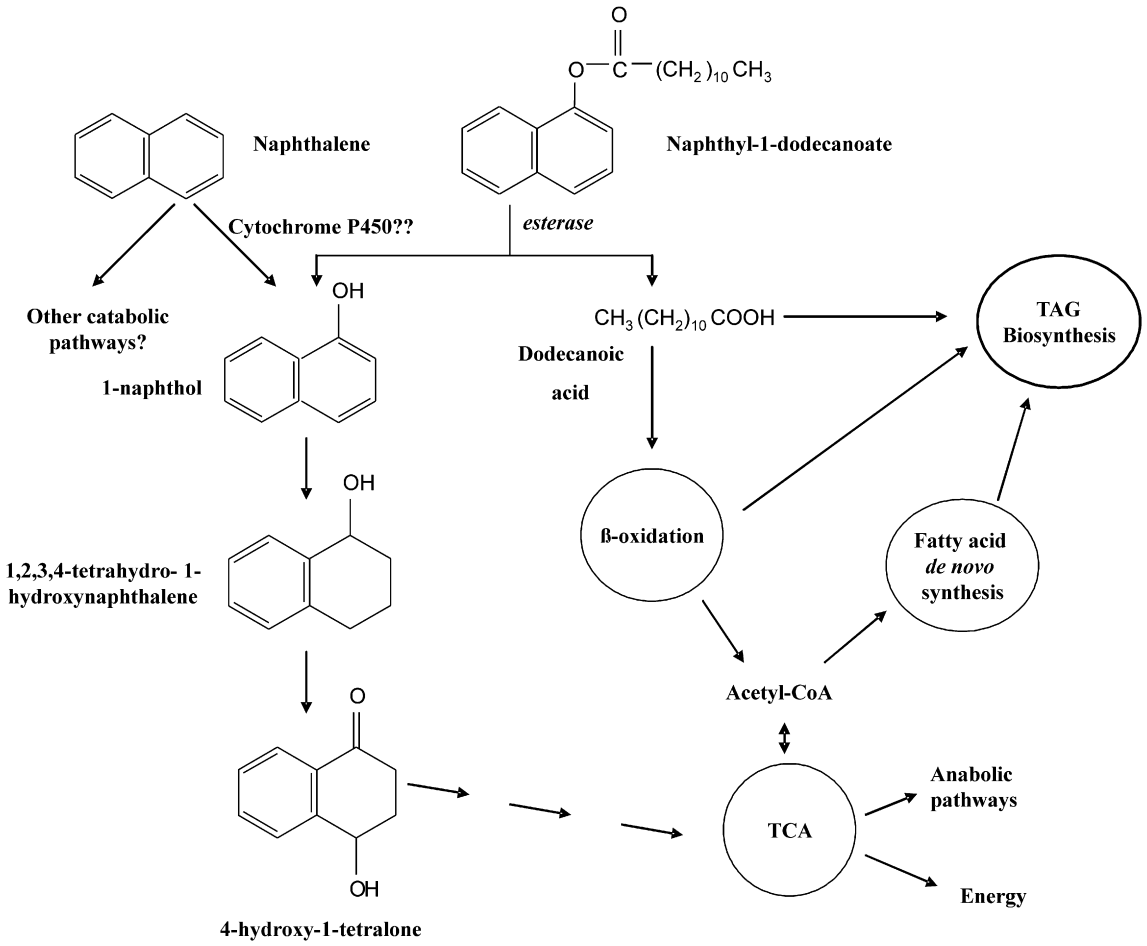


Fig. 3. Proposed pathways for the metabolism of naphthalene and naphthyl-1-dodecanoate by *Rhodococcus* sp. 602 under N-starved conditions.

pyridine (1:1, v/v; 60 °C for 30 min) before GC–MS analyses. Mass spectra of lipids were obtained using a HP6890 gas chromatograph interfaced to a MD800 Voyager mass spectrometer.

The GC–MS was configured with: on-column injection; a DB-5MS column (30 m × 0.25 mm × 0.25 μm); temperature programming (60 °C hold for 1 min, from 60 to 130 °C at 20 °C min^{-1}, from 130 to 310 °C at 4 °C min^{-1} followed by a 30 min hold at 310 °C); helium carrier gas (constant flow of 0.8 ml min^{-1}); 50 eV electron impact (EI) ionization with a source temperature of 200 °C.

2.7. Nucleotide sequence accession numbers

Nucleotide sequences determined in this work have been deposited in the GenBank database under accession numbers FJ768000 (16S rRNA gene, *Rhodococcus* sp. 602) and FJ790216 (*gyr*B gene, *Rhodococcus* sp. 602).

3. Results

3.1. Isolation and characterization of the indigenous strain 602

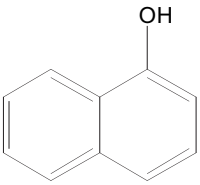
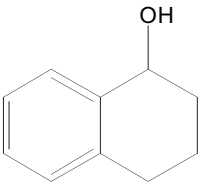
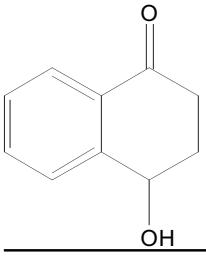
Strain 602 was selected among other isolates by a screening colony staining method based on the use of Sudan Black B to determine the occurrence of lipid inclusion bodies in the cytoplasm. The colonies of strain 602 were strongly stained by the lipophilic dye after cultivation on gluconate under nitrogen-limiting conditions. This strain was an aerobic, non-motile and Gram-positive bacterium. Cells were rods during exponential-growth phase but formed short rods or cocci at stationary-growth phase. Colonies were opaque and convex with regular edges, and showed a salmon-pink pigmentation in all media utilized. In addition, it showed catalase, urease and DNase activities but no oxidase activity. Cells were able to grow under a broad range of conditions: pH between 5 and 10, temperature between 8 and 37 °C, and salinity up to 5% NaCl (w/v).

3.2. Phylogenetic characterization of strain 602

A phylogenetic analysis based on 16S rDNA sequence (1451 bp) was performed to identify strain 602. Comparison of its sequence in the GenBank database showed 99% similarity to *Rhodococcus jostii* strain RHA1. A similar result (97% of similarity with strain RHA1) was observed when analyzing 414 bp corresponding to the *gyr*B gene (DNA gyrase B subunit).

Both partial sequences were compared with those of *Rhodococcus* species and representatives of other mycolic-acid-containing actinomycete genera. The phylogenetic analyses performed showed that strain 602 is closely related to strain

Table 4
Metabolites detected after cultivation of *Rhodococcus* sp. strain 602 on naphthalene and naphthyl-1-dodecanoate in the absence of the nitrogen source.

Structure	MW (silylated)	t_R (min)	Compound
	216	9.58	1-Naphthol
	220	9.64	1,2,3,4-Tetrahydro-1-hydroxynaphthalene
	234	11.58	4-Hydroxy-1-tetralone

Abbreviations: MW, molecular weight; t_R, retention time.

RHA1 (Fig. 1), and both fell into the '*Rhodococcus erythropolis*' subgroup mentioned by Gürtler et al. (2004) (Fig. 1). The topologies of the trees inferred by maximum-likelihood, maximum-parsimony, and neighbor-joining methods were congruent.

3.3. Utilization of diverse carbon sources by strain 602

Strain 602 possesses the ability to utilize several compounds as sole carbon source. Cells were able to grow on different sugars (glucose, lactose, sucrose), organic salts (acetate, benzoate, gluconate, valerate), *n*-alkanes (C_5–C_{18}), alcohols such as ethanol or 2-propanol, aromatic compounds (benzene, toluene, xylene, naphthalene, phenanthrene, fluorene, anthracene) and substituted organic acids (alkyl benzene sulfonic acid). The activity of strain 602 on different carbon sources was further investigated by monitoring respiration (CO_2 production). After 7 days of incubation, the maximum values of CO_2 released were obtained from sodium benzoate, 3-cyclohexylpropionic acid and *n*-hexadecane (1.36, 1.20 and 0.89 mmol l^{-1}, respectively). Lower mineralization values were obtained from PAH, such as naphthalene, phenanthrene and naphthyl-1-dodecanoate (0.17, 0.23 and 0.39 mmol l^{-1}, respectively), or compounds as glycerol or octadecylamine (0.45 and 0.26 mmol l^{-1}, respectively).

3.4. Accumulation of triacylglycerols by Rhodococcus sp. 602 under nitrogen-limiting conditions

The accumulation of polyhydroxyalkanoates (PHA), TAG and WE during cultivation of strain 602 in the presence of different carbon sources under nitrogen-limiting conditions was investigated. Nitrogen limitation promotes the biosynthesis and accumulation of lipids by bacteria (Alvarez, 2006). Like *R. opacus* PD630 and *R. jostii* RHA1 (Alvarez et al., 1996; Hernandez et al., 2008), strain 602 was able to accumulate significant amounts of TAG and minor amounts of PHA when cells were cultivated on gluconate, benzoate or *n*-hexadecane under nitrogen-limiting conditions, as revealed by TLC (data not shown) and GC analyses (Table 2). No WE were detected by TLC analysis (data not shown). Gluconate- and benzoate-grown cells contained saturated and monounsaturated long-chain (from C_{14} to C_{18}) fatty acids as main fatty acid residues, whereas C_{14} and C_{16} fatty acids predominated in *n*-hexadecane-grown cells (Table 2).

A partial sequence (1386 bp) corresponding to a putative WS/DGAT of strain 602 was amplified using primers designed for *atf1* gene from *R. opacus* PD630 (Alvarez, 2009). The amino acid sequence deduced shared conserved sequences, including the putative active-site motif HHxxxDG (HHSLTDG in strain 602) described for these enzymes in other TAG-accumulating bacteria. The sequence showed the highest similarities (94% and 87% identity) to Atf1 protein of *R. opacus* strain PD630 (Alvarez et al., 2008) and to the predicted protein Atf3 (*ro00039*, homologous to *atf1* from *R. opacus* PD630) of *R. jostii* strain RHA1 (Hernandez et al., 2008).

3.5. Synthesis and accumulation of triacylglycerols from naphthyl compounds by resting cells of Rhodococcus sp. 602

TLC-Iatroscan analysis was used to determine the different lipidic compounds produced by resting cells of strain 602

incubated in the presence of naphthalene or naphthyl-1-dodecanoate and in the absence of a nitrogen source (Table 3). The total lipid fraction of naphthalene- and naphthyl-1-dodecanoate-grown cells represented ca. 6.9% and 15.3% of cellular dry weight (CDW), respectively. The TAG fraction represented 48.8% of the total lipid extract (by dry weight) in cells cultivated on naphthalene, while only 7.4% of TAG were detected in cells cultivated on naphthyl-1-dodecanoate (Table 3). Several other compounds such as alcohols or polar lipids were also identified in cells of both cultures (Table 3).

Three fractions (neutral lipids, glycolipids and phospholipids) were obtained by column chromatography of an aliquot of the total lipid extract. GC–MS analyses of the first fraction confirmed the presence of TAG in cells cultivated on naphthalene as well as on naphthyl-1-dodecanoate. No compound with MW and chemical structure compatible to WE were detected during these analyses. Similar to the fatty acid composition of lipids accumulated by gluconate-grown cells, TAG produced by resting cells exposed to naphthalene contained odd- and even-numbered linear fatty acids with carbon chain lengths ranging from 14 to 18 carbon atoms. On the other hand, TAG from cells incubated with naphthyl-1-dodecanoate contained a mixture of short-chain (C_8, C_{10}, C_{12}) fatty acids (Fig. 2).

3.6. Other major metabolites of naphthyl-compound oxidation

In addition to the aforementioned lipids, some oxidation products of naphthalene and naphthyl-1-dodecanoate were detected during GC–MS analyses (Table 4). The identification of these metabolites was based on the interpretation of their mass spectra and on the comparison of their fragmentation characteristics with data from the literature and from mass spectra databases. 1-Naphthol was the main intermediate detected in cells, while 1,2,3,4-tetrahydro-1-hydroxynaphthtalene and 4-hydroxy-1-tetralone appeared in minor amounts (Table 4).

4. Discussion

The indigenous Gram-positive bacterium strain 602 belonging to the *Rhodococcus* genus was isolated from a polluted soil sample collected in Patagonia. Phylogenetic analyses demonstrated that strain 602 is close to *R. jostii* RHA1, a potent polychlorinated biphenyl-degrading actinomycete (McLeod et al., 2006).

Like most rhodococci, the indigenous strain 602 possesses the ability to degrade a broad spectrum of carbon sources and to accumulate significant amounts of TAG. In this context, Hernandez et al. (2008) recently reported on the capacity of *R. jostii* RHA1 to synthesize TAG as main intracellular storage compounds. This microorganism was showed to contain 14 genes encoding putative WS/DGAT enzymes likely involved in TAG biosynthesis (Hernandez et al., 2008). Alvarez et al. (2008) identified 10 genes encoding putative WS/DGAT enzymes in *R. opacus* PD630, which is another bacterium taxonomically related to strain 602. Among these genes, *atf1* contributes significantly to the TAG accumulation in strain PD630. This gene has been cloned and characterized at the molecular level (Alvarez et al., 2008). In this study, we partially amplified the gene encoding a putative WS/DGAT enzyme from strain 602, which is homologous to *atf1* from *R. opacus* PD630 and *atf3* from *R. jostii* RHA1. It has been demonstrated that prokaryotic WS/DGAT's are very promiscuous enzymes accepting a wide range of substrates, which are incorporated into lipids of diverse chemical structures (Kalscheuer et al., 2003; Stöveken et al., 2005; Uthoff et al., 2005). The flexibility of the TAG and WE biosynthetic pathways has been demonstrated in vivo in members of actinomycete bacteria (Alvarez et al., 2001, 2002; Silva et al., 2007). We thus investigated the ability of the indigenous *Rhodococcus* sp. strain 602 to produce TAG and/or WE from naphthyl compounds.

Strain 602 was able to synthesize TAG from naphthalene and naphthyl-1-dodecanoate during incubation of cells in mineral medium devoid of a nitrogen source (Fig. 2 and Table 3). To our knowledge, this is the first report on the formation of TAG in polyaromatic hydrocarbon-degrading bacteria. Neither TAG nor WE containing residues with polyaromatic structures were detected in this study. TAG produced by resting cells cultivated on naphthalene contained C-even and C-odd linear fatty acids ranging from C_{14} to C_{18}, which may be de novo synthesized after hydrocarbon catabolism. This anabolic route is usually utilized by actinomycetes to synthesize TAG with similar fatty acid compositions (Alvarez, 2006). On the other hand, resting cells cultivated on naphthyl-1-dodecanoate produced significant proportions of 1-naphthol and dodecanoic acid, and a mixture of novel TAG containing only short-chain fatty acids (C_8, C_{10} and C_{12}). This indicated the initial hydrolysis of the ester bond of the substrate by an esterase and subsequent β-oxidation of dodecanoic acid into shorter chain fatty acids (Figs. 2 and 3). Dodecanoic acid and the derived fatty acids were then incorporated into TAG.

The detection of 1-naphthol after cultivation of cells on naphthalene indicated that strain 602 could degrade naphthalene by a mono-oxygenase such as P450 cytochrome (Table 4 and Fig. 3), as was previously proposed for fungi (Ferris et al., 1976). The occurrence of cytochrome P450-dependent monooxygenases has been previously reported for *Rhodococcus* genus (Gürtler et al., 2004; Larkin et al., 2005).

Our results also indicated that strain 602 further oxidized 1-naphthol sequentially to 1,2,3,4-tetrahydro-1-hydroxynaphthtalene and to 4-hydroxy-1-tetralone (Table 4 and Fig. 3), by hydroxylations and reductions of the aromatic ring. The tetralone could be subsequently oxidized through the tricarboxylic acid cycle (Fig. 3). Similar biodegradation pathways of 1-naphthol have been described in other microorganisms and eukaryotic cells, including two bacterial strains of *Pseudomonas* and *Streptomyces* (Gopishetty et al., 2007; Samanta et al., 1999). To our knowledge, this is the first report of the involvement of the 1-naphthol pathway in degradation of naphthalene by a member of the *Rhodococcus* genus. Other metabolites were not detected in this study;

however, the existence of alternative catabolic pathways of naphthyl compounds in strain 602 cannot be ruled out.

In conclusion, our results provide an insight into the metabolism of naphthyl compounds by *Rhodococcus* sp. 602 under nitrogen-limiting conditions. The ability of this strain to maintain metabolic activity and to convert hydrocarbons into lipids in the absence of a nitrogen source, as occurs in naturally poor environments, could be relevant for predicting its use in bioremediation of polluted soil.

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References

Alvarez, A.F., 2009. Aspectos moleculares de la acumulación de triacilglicéridos en *Rhodococcus opacus* PD630. Ph.D. thesis, Universidad Nacional de la Patagonia San Juan Bosco, Comodoro Rivadavia, Argentina.

Alvarez, A.F., Alvarez, H.M., Kalscheuer, R., Wältermann, M., Steinbüchel, A., 2008. Cloning and characterization of a gene involved in triacylglycerol biosynthesis and identification of additional homologous genes in the oleaginous bacterium *Rhodococcus opacus* PD630. Microbiology 154, 2327–2335.

Alvarez, H.M., 2006. Bacterial triacylglycerols. In: Welson, L.T. (Ed.), Triglycerides and Cholesterol Research. Nova Science Publishers, Inc., Hauppauge, New York, pp. 159–176.

Alvarez, H.M., Mayer, F., Fabritius, D., Steinbüchel, A., 1996. Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. Arch. Microbiol. 165, 377–386.

Alvarez, H.M., Souto, M.F., Viale, A., Pucci, O.H., 2001. Biosynthesis of fatty acids and triacylglycerols by 2,6,10,14-tetramethyl pentadecane-grown cells of *Nocardia globerula* 432. FEMS Microbiol. Lett. 200, 195–200.

Alvarez, H.M., Luftmann, H., Silva, R.A., Cesari, A.C., Viale, A., Wältermann, M., Steinbüchel, A., 2002. Identification of phenyldecanoic acid as a constituent of triacylglycerols and wax ester produced by *Rhodococcus opacus* PD630. Microbiology 148, 1407–1412.

Annweiler, E., Richnow, H.H., Antranikian, G., Hebenbrock, S., Garms, C., Franke, S., Francke, W., Michaelis, W., 2000. Naphthalene degradation and incorporation of naphthalene-derived carbon into biomass by the thermophile *Bacillus thermoleovorans*. Appl. Environ. Microbiol. 66, 518–523.

Augustin, T., Schlosser, D., Baumbach, R., Schmidt, J., Grancharov, K., Krauss, G., Krauss, G.-J., 2006. Biotransformation of 1-naphthol by a strictly aquatic fungus. Curr. Microbiol. 52, 216–220.

Bosch, R., García-Valdés, E., Moore, E.R.B., 2000. Complete nucleotide sequence and evolutionary significance of a chromosomally encoded naphthalene-degradation lower pathway from *Pseudomonas stutzeri* AN10. Gene 245, 65–74.

Caradec, S., Grossi, V., Gilbert, F., Guigue, C., Goutx, M., 2003. Influence of various redox conditions on the degradation of microalgal triacylglycerols and fatty acids in marine sediments. Org. Geochem. 35, 277–287.

Cerniglia, C.E., Gibson, D.T., 1977. Metabolism of naphthalene by *Cunninghamella elegans*. Appl. Environ. Microbiol. 34, 363–370.

Cerniglia, C.E., Gibson, D.T., Van Baalen, C., 1980. Oxidation of naphthalene by cyanobacteria and microalgae. J. Gen. Microbiol. 116, 495–500.

Cerniglia, C.E., Freeman, J.P., Evans, F.E., 1984. Evidence for an arene oxide-NIH shift pathway in the transformation of naphthalene to 1-naphthol by *Bacillus cereus*. Arch. Microbiol. 138, 283–286.

Di Gennaro, P., Rescalli, E., Galli, E., Sello, G., Bestetti, G., 2001. Characterization of *Rhodococcus opacus* R7, a strain able to degrade naphthalene and *o*-xylene isolated from a polycyclic aromatic hydrocarbon-contaminated soil. Res. Microbiol. 152, 641–651.

Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17, 368–376.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Felsenstein, J., 1993. Phylip: Phylogeny Inference Package, Version 3.67. University of Washington, Seattle, USA.

Ferris, J.P., MacDonald, L.H., Patrie, M.A., Martin, M.A., 1976. Aryl hydrocarbon hydroxylase activity in the fungus *Cunninghamella bainieri*: evidence for the presence of cytochrome P-450. Arch. Biochem. Biophys. 175, 443–452.

Fredrickson, J.K., Brockman, F.J., Workman, D.J., Li, S.W., Stevens, T.O., 1991. Isolation and characterization of a subsurface bacterium capable of growth on toluene, naphthalene, and other aromatic compounds. Appl. Environ. Microbiol. 57, 796–803.

Gopishetty, S.R., Heinemann, J., Deshpande, M., Rosazza, J.P.N., 2007. Aromatic oxidations by *Streptomyces griseus*: biotransformations of naphthalene to 4-hydroxy-1-tetralone. Enzyme Microb. Technol. 40, 1622–1626.

Grund, E., Denecke, B., Eichenlaub, R., 1992. Naphthalene degradation via salicylate and gentisate by *Rhodococcus* sp. strain B4. Appl. Environ. Microbiol. 58, 1874–1877.

Gürtler, V., Mayall, B.C., Seviour, R., 2004. Can whole genome analysis refine the taxonomy of the genus *Rhodococcus*? FEMS Microbiol. Rev. 28, 377–403.

Hernandez, M.A., Mohn, W.W., Martinez, E., Rost, E., Alvarez, A.F., Alvarez, H.M., 2008. Biosynthesis of storage compounds by *Rhodococcus jostii* RHA1 and global identification of genes involved in their metabolism. BMC Genomics 9, 600–613.

Kalscheuer, R., Uthoff, S., Luftmann, H., Steinbüchel, A., 2003. *In vitro* and *in vivo* biosynthesis of wax diesters by an unspecific bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase from *Acinetobacter calcoaceticus* ADP-1. Eur. J. Lipid Sci. Technol. 105, 578–584.

Kluge, A.G., Farris, F.S., 1969. Quantitative phyletics and the evolution of the anurans. Syst. Zool. 18, 1–32.

Larkin, M.J., Kulakov, L.A., Allen, C.C.R., 2005. Biodegradation and *Rhodococcus* – masters of catabolic versatility. Curr. Opin. Biotechnol. 16, 282–290.

Martínková, L., Uhnáková, B., Pátek, M., Nešvera, J., Křen, V., 2009. Biodegradation potential of the genus *Rhodococcus*. Environ. Int. 35, 162–177.

McLeod, M.P., Warren, R.L., Hsiao, W.W.L., Araki, N., Myhre, M., Fernandes, C., Miyazawa, D., Wong, W., Lillquist, A.L., Wang, D., Dosanjh, M., Hara, H., Petrescu, A., Morin, R.D., Yang, G., Stott, J.M., Schein, J.E., Shin, H., Smailus, D., Siddiqui, A.S., Marra, M.A., Jones, S.J. M., Holt, R., Brinkman, F.S.L., Miyauchi, K., Fukuda, M., Davies, J.E., Mohn, W.W., Eltis, L.D., 2006. The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. Proc. Natl. Acad. Sci. U S A 103, 15582–15587.

Nicholas, K.B., Nicholas Jr., H.B., 1997. GeneDoc: analysis and visualization of genetic variation. Version 2.7.000. Distributed by the author.

Notredame, C., Higgins, D., Heringa, J., 2000. T-Coffee: a novel method for multiple sequence alignments. J. Mol. Biol. 302, 205–217.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Samanta, S.K., Chakraborti, A.K., Jain, R.K., 1999. Degradation of phenanthrene by different bacteria: evidence for novel transformation sequences involving the formation of 1-naphthol. Appl. Microbiol. Biotechnol. 53, 98–107.

Schlegel, H.G., Kaltwasser, H., Gottschalk, G., 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. 38, 209–222.

Schlegel, H.G., Lafferty, R., Krauss, I., 1970. The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch. Mikrobiol. 71, 283–294.

Silva, R.A., Grossi, V., Alvarez, H.M., 2007. Biodegradation of phytane (2, 6, 10, 14-tetramethylhexadecane) and accumulation of related isoprenoid

wax esters by *Mycobacterium ratisbonense* strain SD4 under nitrogen-starved conditions. FEMS Microbiol. Lett. 272, 220–228.

Stöveken, T., Kalscheuer, R., Malkus, U., Reichelt, R., Steinbüchel, A., 2005. The wax ester synthase/acyl coenzyme A:diacylglycerol acyltransferase from *Acinetobacter* sp. strain ADP1: characterization of a novel type of acyltransferase. J. Bacteriol. 187, 1369–1376.

Taki, H., Syutsubo, K., Mattison, R., Harayama, S., 2007. Identification and characterization of *o*-xylene-degrading *Rhodococcus* sp. which were dominant species in the remediation of *o*-xylene-contaminated soils. Biodegradation 18, 17–26.

Uthoff, S., Stöveken, T., Weber, N., Vosmann, K., Klein, E., Kalscheuer, R., Steinbüchel, A., 2005. Thio wax ester biosynthesis utilizing the unspecific bifunctional wax ester synthase/acyl coenzyme A:diacylglycerol acyltransferase of *Acinetobacter* sp. strain ADP1. Appl. Environ. Microbiol. 71, 790–796.

Uz, I., Duan, Y.P., Ogram, A., 2000. Characterization of the naphthalene-degrading bacterium, *Rhodococcus opacus* M213. FEMS Microbiol. Lett. 185, 231–238.

Waidyanatha, S., Zheng, Y., Serdar, B., Rappaport, S.M., 2004. Albumin adducts of naphthalene metabolites as biomarkers of exposure to polycyclic aromatic hydrocarbons. Cancer Epidemiol. Biomarkers Prev. 13, 117–124.

Warhurst, A.M., Fewson, C.A., 1994. Biotransformations catalyzed by the genus *Rhodococcus*. Crit. Rev. Biotechnol. 14, 29–73.

Zhou, N.-Y., Fuenmayor, S.L., Williams, P.A., 2001. *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. J. Bacteriol. 183, 700–708.