Appl Microbiol Biotechnol (2015) 99:4071–4084
DOI 10.1007/s00253-014-6320-4

ENVIRONMENTAL BIOTECHNOLOGY

Enrichment of aliphatic, alicyclic and aromatic acids by oil-degrading bacteria isolated from the rhizosphere of plants growing in oil-contaminated soil from Kazakhstan

Annett Mikolasch · Anel Omirbekova · Peter Schumann · Anne Reinhard · Halah Sheikhany · Ramza Berzhanova · Togzhan Mukasheva · Frieder Schauer

Received: 2 October 2014 / Revised: 9 December 2014 / Accepted: 12 December 2014 / Published online: 16 January 2015

Abstract Three microbial strains were isolated from the rhizosphere of alfalfa (*Medicago sativa*), grass mixture (*Festuca rubra*, 75 %; *Lolium perenne*, 20 %; *Poa pratensis*, 10 %), and rape (*Brassica napus*) on the basis of their high capacity to use crude oil as the sole carbon and energy source. These isolates used an unusually wide spectrum of hydrocarbons as substrates (more than 80), including *n*-alkanes with chain lengths ranging from C_{12} to C_{32}, monomethyl- and monoethyl-substituted alkanes (C_{12}–C_{23}), *n*-alkylcyclo alkanes with alkyl chain lengths from 4 to 18 carbon atoms, as well as substituted monoaromatic and diaromatic hydrocarbons. These three strains were identified as *Gordonia rubripertincta* and *Rhodococcus* sp. SBUG 1968. During their transformation of this wide range of hydrocarbon substrates, a very large number of aliphatic, alicyclic, and aromatic acids was detected, 44 of them were identified by GC/MS analyses, and 4 of them are described as metabolites for the first time. Inoculation of plant seeds with these highly potent bacteria had a beneficial effect on shoot and root development of plants which were grown on oil-contaminated sand.

Keywords Aliphatic acids · Alicyclic acids · Aromatic acids · Oil components · Rhizoremediation · Degradation

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-6320-4) contains supplementary material, which is available to authorized users.

A. Mikolasch (✉) · A. Reinhard · F. Schauer
Department of Applied Microbiology, Institute of Microbiology, University Greifswald, Friedrich-Ludwig-Jahn-Str. 15, 17487 Greifswald, Germany
e-mail: annett.mikolasch@uni-greifswald.de

A. Omirbekova · R. Berzhanova · T. Mukasheva
Department of Biology and Biotechnology, Al-Farabi Kazakh National University, Building 6, Al-Farabi Ave. 71, 050040 Almaty, Kazakhstan

P. Schumann
Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany

H. Sheikhany
Department of Basic Science, Faculty of Agriculture, Damascus University, Damascus, Syria

Introduction

Quantitatively, oil and oil products are the main environmental pollutants in the world. Exploitation of oil deposits is inevitably accompanied by accidents during extraction, transportation, and storage, and these can lead to the pollution of soil and water. Pollutants are naturally removed by evaporation, photochemical and geochemical reactions, and biodegradation (Komukai-Nakamura et al. 1996; Tjessem and Aaberg 1983).

Bioremediation uses microbes or other biological systems to degrade environmental pollutants (Atlas 1991; Atlas and Atlas 1991; Dua et al. 2002; Heipieper 2007; Owsianiak et al. 2009; Song et al. 1990; Wang and Bartha 1990). Rhizoremediation, which can be used at the final stages of polluted soil re-cultivation, is based on the use of microorganisms to utilize environmental pollutants as carbon and energy source and leads to the destruction of organic pollutants by microorganisms associated with plant roots (Gerhardt et al. 2009; Kuiper et al. 2004). The action of good root-colonizing and pollutant-degrading bacterial strains can result in efficient cleaning of polluted soils. It has been reported that root systems of some plants such as tall fescue, sorghum, maize, alfalfa, ryegrass, Bermuda grass, and rice contain highly potent hydrocarbon-degrading microorganisms (Graj et al. 2013; Hong et al. 2011; Maila et al. 2005; Soleimani et al. 2010).

To elucidate the degrading potential of microorganisms in oil-contaminated soil from Kazakhstan, the present study focused on the isolation and characterization of oil-degrading microorganisms from the rhizosphere of alfalfa (*Medicago sativa*), grass mixture (*Festuca rubra*, 75 %; *Lolium perenne*, 20 %; *Poa pratensis*, 10 %), and rape (*Brassica napus*) in Kazakhstan and on the structural characterization of a very large number of aliphatic, alicyclic, and aromatic acids produced during the transformation of a wide range of the hydrocarbon components of crude oil. In order to understand the mechanisms by which the microorganisms degrade the oil components, the isolated species have been cultivated in liquid medium in the presence of oil as sole carbon and energy source, and the supernatants were analyzed chromatographically to separate a wide variety of metabolites identified by GC/MS. The oil degradation, the results of the chemical characterization of a very large number of aliphatic, alicyclic, and aromatic acid products, and the potential of oil decomposition by the isolated species are presented in comparison with the results of well-described species isolated from oil-polluted sand samples collected in the Saudi Arabian Desert and from sewage sludge in Germany. These results are discussed in the context of the shoot and root development of plants which were grown on oil-contaminated sand after inoculation of the seeds with these highly potent newly isolated bacteria.

Materials and methods

Media

Nutrient broth II (SIFIN, Berlin) served as cultivation medium.

Mineral salts medium (MSM, pH 6.3) containing 1.5 g L^{-1} $(NH_4)_2HPO_4$, 0.7 g L^{-1} KH_2PO_4, 0.8 g L^{-1} $MgSO_4$ $7H_2O$, 0.5 g L^{-1} NaCl, 0.46 g L^{-1} K_2SO_4, and 20 g L^{-1} agar (Hundt et al. 1998) was used for isolation of microorganisms from plant rhizosphere and rhizoplane and, without agar, in liquid form for degradation experiments.

Isolation of microorganisms from plant rhizosphere and rhizoplane

Different microorganism strains were isolated from rhizosphere and rhizoplane of plants after pot growth experiments. Each pot was filled with 5 kg of lightly contaminated soil from the Uzen deposit, Mangystau region, Kazakhstan. Soil was analyzed for total content of oil and was supplemented with oil (Uzen deposit, Mangystau region, Kazakhstan) to achieve a final concentration of 20.0 g kg^{-1}. The pots were planted with alfalfa (*Medicago sativa*), grass mixture (*Festuca rubra*, 75 %; *Lolium perenne*, 20 %; *Poa pratensis*, 10 %), and rape (*Brassica napus*). The plants were grown in a growth room at RT with 14 h of light period and 10 h of dark period. Duration of cultivation was 50 days.

The plants were removed from the pots, and the bulk soil was shaken off the roots. Ten grams of root sample with adhering soil was washed in 90 mL of sterile distilled water by shaking for 30 min at 25 °C and 220 rpm (Upadhyay et al. 2009). After shaking, 1 mL of the suspension was added to a tube with 9 mL sterile distilled water. This dilution step was repeated three times. The last suspension was used for isolation of rhizosphere microorganisms. The root sample taken from the first flask was washed again in 90 mL of sterile distilled water by shaking for 30 min at 25 °C and 220 rpm; this suspension was also used to isolate microorganisms. The microorganisms of these latter samples were designated as being derived from the rhizoplane of plants.

For isolation of hydrocarbon-oxidizing microorganisms, the plating dilution technique was used. The suspensions were plated on MSM medium. Ten milliliters per liter of oil was added as sole source of carbon and energy.

Identification of isolated microorganisms

Cell morphology (shape, size) of the isolates was determined using a phase-contrast microscope (Axiolab, Zeiss). The Gram characteristics of all bacteria were determined using the KOH test (Suslow et al. 1982).

The three isolates were identified first by their whole cellular fatty acid profiles and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra. Biomass of strains grown of Tryptic Soy Agar (medium 535; www.dsmz.de) at 28 °C for 2 days was used for extraction of cellular fatty acids. Cellular fatty acids were analyzed by gas chromatography using the Microbial Identification System (MIDI, Sherlock version 6.1; method, TSBA40; gas chromatograph, model 6890 N, Agilent Technologies) following the method of Stead et al. (1992). Biomass for MALDI-TOF mass spectrometry (MS) analysis was collected after cultivating the strains on Caso Agar (medium 220; www.dsmz.de) at 30 °C for 24 h. Whole-cell protein extracts were obtained according to Protocol 3 of Schumann and Maier (2014) and analyzed by using a Microflex L20 mass spectrometer (Bruker Daltonics). The MALDI-TOF mass spectra were identified with the MALDI-Biotyper software (version 3.1, Bruker Daltonics) using the database update V4.0.0.1.

Molecular genetic analysis for the identification of isolated strains was performed by the National Scientific Shared Laboratory of Biotechnology of National Center for Biotechnology, Kazakhstan. The chromosomal DNA of strains was isolated by the Kate Wilson method (Clayton et al. 1995). Identification of bacteria was performed by direct determination of the nucleotide sequence of the 16S

recombinant RNA (rRNA) gene fragment, followed by a BLAST search analysis carried out on the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim et al. 2012).

Amplification of 16S rRNA fragments

The PCR reaction was performed with universal primers 8 F 5′-AgAgTTTgATCCTggCTCAg-806R-3 and the 5′-ggACTACCAgggTATCTAAT for a total volume of 20 μL. The PCR mixture contained 150 ng of DNA, 1 unit of Maxima Hot Start Taq DNA Polymerase (Fermentas), 0.2 mM of each dNTP, 1× PCR buffer (Fermentas), 2.5 mM $MgCl_2$, and 10 pmol of each primer. The PCR amplification program included long denaturation at 95 °C for 7 min; 35 cycles at 95 °C for 15 s; 52 °C for 30 s; 72 °C for 30 s; and final elongation at 72 °C for 7 min. The PCR program was carried out in a thermocycler GeneAmp PCR System 9700 (Applied Biosystems).

Determination of nucleotide sequence

Purification of PCR products from unrelated primers was performed using the Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas) (Werle et al. 1994).

Sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions followed by separation of the fragments on an automated Genetic Analyzer 3730 xl DNA Analyzer (Applied Biosystems).

Reference strains

Nocardia cyriacigeorgica SBUG 1472 was originally isolated from oil-polluted sand samples collected in the Saudi Arabian Desert by enrichment cultivation (Nhi-Cong et al. 2010) and deposited in the strain collection of the Department of Biology of the University Greifswald.

Rhodococcus erythropolis SBUG 271 was originally isolated from sewage sludge collected in Germany by enrichment cultivation (Stope et al. 2002) and deposited in the strain collection of the Department of Biology of the University Greifswald.

Evaluation of growth in the presence of crude oil

Cultivation of bacteria was carried out in 250-mL flasks containing 50 mL of MSM (pH 6.3) supplemented with 1.5 mL of oil. Crude oil from the Uzen deposit, Mangystau region, Kazakhstan, diesel fuel, fuel oil, and gasoline were used as the sole carbon and energy sources at 25 °C and 220 rpm. Assays without substrate and with nutrient broth were used as controls. The dry weight of cultures (biomass grams per liter) was determined after 6 and 10 days of growth by filtering the whole cultures through a glass fiber filter (Whatmann®, Dassel, Germany) which was then dried for 2 h at 100 °C.

Evaluation of growth in the presence of aromatic hydrocarbons

Cultivation of bacteria was carried out on biphenyl and naphthalene. Biphenyl dissolved in diethyl ether was added to sterile 500-mL Erlenmeyer flasks to a final concentration of 100 mg mL^{-1}, naphthalene as a diethyl ether solution to a final concentration of 20 mg mL^{-1}. After evaporation of the diethyl ether over 12 h, 100 mL of MSM was added to each flask, and flasks were shaken for 24 h at 30 °C and 180 rpm to achieve saturation of the compound in the liquid medium. A cell suspension was then added until an optical density (A 500) of 0.04 was reached. Cultures were incubated with biphenyl or naphthalene on a rotary shaker at 30 °C and 180 rpm. Flasks with cell suspension in nutrient broth were used as controls. The mass of cells was determined by the dry weight method for inhomogeneous cell suspensions and by the optical density method for homogeneous cell suspensions.

Degradation experiments

Cultures were shaken in 500-mL Erlenmeyer flasks with 100 mL MSM medium and 1 mL crude oil (Uzen deposit, Mangystau region, Kazakhstan) at 30 °C and 180 rpm. Assays without oil substrate or without cells or with cells and 1 % glucose as sole source of carbon and energy were used as controls.

After 6 days of incubation, the reaction was stopped, the pH value was measured with pH paper, and 5 μL of phenyloctane was added as internal standard. The whole cultures of such transformation assays were first adjusted to pH 9.0 and extracted three times with half a volume diethyl ether. The extracted supernatant was then adjusted to pH 2.0 and extracted further three times with half a volume diethyl ether. The organic phases were dried over anhydrous sodium sulfate and concentrated by rotary evaporation. The residues obtained were dried under a gentle nitrogen stream and dissolved in hexane before analysis. The data are reported as means for two separate experiments with replicated batch cultures. The standard deviation in these replicates was no more than 10 %.

Chemical analysis and identification of products

Gas chromatography–mass spectrometry (GC–MS)

The oil components and metabolites were detected and quantified by injecting 1 μL of the extract into an Agilent gas

chromatograph 7890A GC System (Waldbronn, Germany) equipped with a capillary column (Agilent 1901 S-433, 30 m×250 μm×0.25 μm, HP-5 ms column) and a mass selective detector 5975C inert XL EI/CI MSD with a quadrupole mass spectrometer. The injector was operated at 250 °C, and the injection was pulsed splitless at 10.5 psi for 2 min. For the analysis of extracts after alkaline extraction, we used elution profile I: the column temperature started at 40 °C and increased to 310 °C at 8 °C min^{-1} and was finally maintained at 310 °C for 6.25 min. For the analysis of extracts after acidic extraction, we used elution profile II: the column temperature started at 40 °C and increased to 310 °C at 4 °C min^{-1} and was finally maintained at 310 °C for 7.5 min. To analyze the acid-extractable products, the extracts after acidic extraction were derivatized by methylation with diazomethane as described by De Boer and Backer (1956) in a microapparatus (Aldrich-Chemie, Steinheim, Germany). The mass spectrometer conditions were set to 250 °C for the interface; the source conditions were 230 °C; and the quadrupole temperature was set to 150 °C. Analytical standards A and B (A contains C_8–C_{20}, 40 mg L^{-1} each, in hexane; B contains C_{21}–C_{40}, 40 mg L^{-1} each, in hexane; Sigma-Aldrich, Germany) were used to identify aliphatic oil components.

Microbial inoculation of barley seeds by bacteria

Before inoculation, plant seeds were sterilized with 10 % solution of sodium hypochlorite for 30 min and then washed four times with sterile tap water for 2 h. Seeds were laid out on Luria–Bertani agar medium and incubated for 18–20 h at 24 °C for seed sterility control. Inoculation of the seeds with the isolated bacterial strains was carried out by immersing the seeds into a suspension of bacterial cells (1.3×10^8 cells mL^{-1}) for 2–3 h. The inoculated seeds were sown in plastic vessels filled with 150 g of sterile sand mixed thoroughly with sterile oil at a final concentration of 2 %. Assays without oil substrate or without microbial inoculation and without oil substrate were used as controls. For plant mineral nutrition, Murashige and Skoog basal salt macronutrient solution (Sigma-Aldrich) was used. Plants were grown for 10 days with the following regime: 12 h of light/dark periods at 20 °C. The following model systems were used: barley, barley with oil, and barley with oil and inoculated bacterial strain. To study the effects of inoculation, biometric characteristics of the plants were evaluated by measurement of shoot and root lengths.

Results

We isolated 30 strains of microorganisms from the rhizosphere of plants grown on crude oil as the sole source of carbon and energy. Three of these were selected as very powerful degraders of crude oil components. The L-RP20 strain was isolated from the rhizoplane of *Medicago sativa* (alfalfa), T-RP18 from the grass mixture (*Festuca rubra*, 75 %; *Lolium perenne*, 20 %; *Poa pratensis*, 10 %), and R-RZ20 from the rhizosphere of *Brassica napus* (rape).

The Gram-positive isolates were characterized by cellular fatty acid analysis (FAME), MALDI-TOF MS, and 16S rRNA gene fragment analysis. Strain T-RP18 was assigned to the "*R. erythropolis* group" (consisting of *R. erythropolis*, *Rhodococcus globerulus*, *Rhodococcus baikonurensis*, and *Rhodococcus jialingiae*; Táncsics et al. 2014) by all three methods. MALDI-TOF MS differentiated strain T-RP18 clearly from *R. globerulus* (score value only 1.214) but could not differentiate this strain from both *R. erythropolis* (score value 2.091) and *R. baikonurensis* (score value 2.143), two species showing a 16S rRNA gene sequence similarity as high as 99.2 %. *R. jialingiae* (Táncsics et al. 2014) is not yet represented in the MALDI Biotyper database. 16S rRNA gene sequence analysis identified the strains L-RP20 and R-RZ20 as belonging to the genus *Gordonia*. However, the sequences of the partial 16S rRNA gene (676 and 645 bp, respectively) did not allow an unambiguous differentiation of strains L-RP20 and R-RZ20 at the species level. MALDI-TOF MS is well suited for the identification of taxonomically soundly defined species (Schumann and Maier 2014) and identifies both strains L-RP20 and R-RZ20 as *Gordonia rubripertincta* with score values >2.0. This identification result is supported by the FAME analyses which revealed similarity indices of 0.805 (strain L-RP20) and 0.751 (strain R-RZ20) to the "*Gordonia-rubripertincta*-GC subgroup B" as well as by sequence similarities of the partial 16S rRNA gene of 98.1 % (strain L-RP20) and 98.0 % (strain R-RZ20) to the type strain *G. rubripertincta* NBRC 101908^T. The isolated and (tentatively) identified bacteria were deposited at the strain collection of the Department of Biology of the University Greifswald and numbered as *G. rubripertincta* SBUG 1972 (L-RP20), *G. rubripertincta* SBUG 1971 (R-RZ20), and *R. erythropolis*/*R. baikonurensis*/*R. jialingiae* SBUG 1968 (T-RP18, further described as *Rhodococcus* sp. SBUG 1968). The 16S rRNA sequences were deposited in GenBank: *G. rubripertincta* SBUG 1972 (L-RP20) accession no. KM085968, *G. rubripertincta* SBUG 1971 (R-RZ20) accession no. KP233861 and *Rhodococcus* sp. SBUG 1968 (T-RP18) accession no. KP233862.

Growth and crude oil consumption of *G. rubripertincta* SBUG 1971, *G. rubripertincta* SBUG 1972, and *Rhodococcus* sp. SBUG 1968

The dry weight of cultures of *G. rubripertincta* SBUG 1971, *G. rubripertincta* SBUG 1972, and *Rhodococcus* sp. SBUG 1968 increased during cultivation in MSM supplemented with different types of oil as the sole source of carbon and energy. The dry weight of each culture with different oils was

determined at 6 and 10 days (Table 1). After 10 days, all species had grown well on every type of oil with the exception of *G. rubripertincta* SBUG 1972 on fuel oil. However, after 6 days all species showed good growth only on crude oil, while *G. rubripertincta* SBUG 1972 also grew on diesel fuel and *G. rubripertincta* SBUG 1971 on fuel oil and gasoline.

To determine the extent of consumption of oil components *G. rubripertincta* SBUG 1971, *G. rubripertincta* SBUG 1972, and *Rhodococcus* sp. SBUG 1968 were incubated in MSM supplemented with 1 mL of crude oil as the sole carbon source. After 6 days of incubation, whole cultures were extracted (after alkalization to pH 9), residues were dissolved in hexane and analyzed by GC/MS [elution profile I] (Supplementary Material Fig. S1).

The volatile *n*-alkanes nonane, decane, undecane, and in part also dodecane evaporated during the incubation process and the following extraction process nearly completely; therefore, these substances could not be analyzed in the elution profile of extracts after alkaline extraction (pH 9) of MSM medium and crude oil. The other *n*-alkanes with chain length from C_{12}–C_{32} were completely degraded by the three strains. The remaining compounds are various branched chain alkanes, aromatics, and heteroaromatic compounds. These results showed that *G. rubripertincta* SBUG 1971 and SBUG 1972 and *Rhodococcus* sp. SBUG 1968 consumed 100 % of alkanes with chain length from C_{12}–C_{32}.

Beside the 24 detected *n*-alkanes 16 *n*-alkylcyclohexanes with *n*-alkyl chain length from C_2 to C_{18} were also shown to be present in the crude oil (Table 2). As described above, for the volatile *n*-alkanes nonane, decane, undecane, and dodecane, the volatile *n*-alkylcyclohexanes ethylcyclohexane, propylcyclohexane, and butylcyclohexane also evaporated during the incubation and subsequent extraction process, so that these substances could not be detected in the elution profile of extracts after alkaline extraction (pH 9) of MSM medium and crude oil. All of the other *n*-alkylcyclohexanes with chain length from C_5 to C_{18} were completely transformed by the species *G. rubripertincta* SBUG 1971 and SBUG 1972 and *Rhodococcus* sp. SBUG 1968. In addition, all alkylcyclopentanes could be transformed by these species.

Furthermore, various *n*-alkyl-methylcyclohexanes, monomethyl- and monoethyl-substituted alkanes, branched chain alkanes, monoaromatics, naphthalenes, and biphenyls were detected in the crude oil—some of these were in low quantity but were clearly identifiable (Table 2). The *n*-alkyl-methylcyclohexanes and methylcyclopentanes, the monomethyl- and monoethyl-substituted alkanes, the alkylmonoaromatics and diaromatics, and the alkylnaphthalenes could not be detected after incubation with the strains *G. rubripertincta* SBUG 1971 and SBUG 1972 and *Rhodococcus* sp. SBUG 1968, whereas the bulk of the branched chain alkanes as well as aromatics and heteroaromatics without alkyl chains remained in the medium.

Analyses of aliphatic, alicyclic, and aromatic acids derived from crude oil components

To characterize the degradation pathways for crude oil components, *G. rubripertincta* SBUG 1971 and SBUG 1972 and *Rhodococcus* sp. SBUG 1968 were incubated in MSM supplemented with 1 mL of crude oil as the sole carbon source. After 6 days of incubation, whole cultures were extracted after acidification to pH 2, and residues were dissolved in hexane, methylated by diazomethane and analyzed by GC/MS [elution profile II] (Supplementary Material Fig. S2).

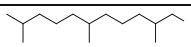
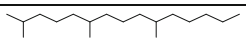
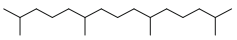
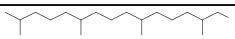
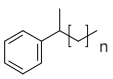
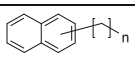
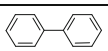
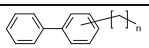
During the incubation of *G. rubripertincta* SBUG 1971 and SBUG 1972 and *Rhodococcus* sp. SBUG 1968 with crude oil, a large number of acidic products were identified by GC/MS analysis by comparison with standard compounds or with the spectral library of the National Institute of Standard Technology (NIST08). In the control extract of the assay with glucose (1 %) as sole source of carbon and in the control extract of the assay with crude oil without cells, no acidic products were detected. In summary, 44 different acidic products were identified by comparison to reference MS spectra of the NIST08 library or to the retention times and mass spectra of standard compounds (overview Table 3; mass spectrum data Supplementary Material Tables S1, S2, S3, S4, S5, and S6). Only one *n*-alkanoic acid was detected in low amount and was identified as heptanoic acid (M1), whereas four monoethyl-substituted and two methyl-

Table 1 Growth experiments with the isolated species *G. rubripertincta* SBUG 1971 (1971), *G. rubripertincta* SBUG 1972 (1972), and *Rhodococcus* sp. SBUG 1968 (1968) on 3 % (*v*/*v*) of different oils after cultivation for 6 and 10 days and controls

Species	Dry weight after growth on different oils (g L^{-1})								Dry weight of controls (g L^{-1})		
	Crude oil		Diesel fuel		Fuel oil		Gasoline		Growth on nutrient broth		Inoculum
	6 days	10 days	6 days	10 days	6 days	10 days	6 days	10 days	6 days	10 days	Start point
1968	3.6 (0.4)	6.6 (0.5)	1.4 (0.8)	4.3 (0.4)	0.3 (0.3)	4.5 (0.5)	1.8 (0.4)	4.3 (0.3)	5.0 (0.2)	5.8 (0.3)	0.9 (0.10)
1971	3.6 (0.5)	7.1 (0.5)	2.1 (0.5)	3.5 (0.1)	3.2 (0.5)	5.6 (0.7)	3.1 (0.1)	4.7 (0.4)	3.5 (0.6)	6.5 (0.5)	0.9 (0.03)
1972	3.6 (0.5)[a]	4.6 (0.2)	3.9 (0.6)	5.8 (0.6)	0.8 (0.4)	2.3 (0.6)	2.4 (0.3)	3.6 (0.3)	5.4 (0.5)	5.7 (0.5)	0.6 (0.05)

[a] Standard deviation

Table 2 Components of crude oil detected by GC/MS and after growth of *Gordonia rubripertincta* SBUG 1971, *Gordonia rubripertincta* SBUG 1972, and *Rhodococcus* sp. SBUG 1968 on crude oil

Detected oil components			**Transformation of oil components** [a]	
Name	*Structure*	*Rf [min]*	*Number of transformed components of the substance group*	*Amount of transformation [%]*
***n*-alkanes** (24 detected compounds)				
nonane to dotriacontane	C_9H_{20} to $C_{32}H_{66}$	5.2 to 33.7	20	100
alkylcyclohexanes				
n-alkylcyclohexanes (16 detected compounds)				
ethylcyclohexane to octadecylcyclohexane	 n= 1 - 17	4.2 to 34.7	13	100
n-alkyl-methylcyclohexanes (6 detected compounds)				
methyl-substituted *n*-alkylcyclohexanes		5.0 to 10.5	6	100
alkylcyclopentanes				
n-alkylcyclopentanes (compounds detected in traces)				
n-alkylcyclopentanes	 n= 5 - x	8.4 to 20.0	6	100
n-alkyl-methylcyclopentanes (compounds detected in traces)				
methyl-substituted *n*-alkylcyclopentanes		8.5 to 20.5	4	100
branched chain alkanes				
monomethyl- and monoethyl-substituted alkanes (20 detected compounds)				
monomethyl- and monoethyl-substituted alkanes	C_9H_{20} to $C_{23}H_{48}$	6.1 to 25.0	14	100
polymethyl-substituted alkanes				
2,6,10-trimethyldodecane		13.7	0	0
2,6,10-trimethylpentadecane		17.8	0	0
pristane (2,6,10,14-tetramethylpentadecane)		18.6	0	0
2,6,10,14-tetramethylhexadecane		20.0	0	0
alkylbenzenes				
n-alkyl-substituted benzenes (three well-detected and traces of longer compounds)	 n = 0-x	4.6 to 20.0	3	100
poly-*n*-alkyl-substituted benzenes (seven well-detected and traces of longer compounds)	 n = 0-x	4.7 to 20.0	7	100
branched-chain-alkyl-substituted benzenes (three well-detected and traces of longer compounds)	 n = 0-x	5.6 to 20.0	3	100
naphthalenes				
naphthalene		10.5	0	0
n-alkyl-substituted naphthalenes (four well-detected and traces of longer alkylnaphthalenes)	 n = 0-x	12.4 to 30.0	4	100
biphenyls				
biphenyl		13.8	0	0
n-alkyl-substituted biphenyls (three well-detected and traces of longer alkylbiphenyls)		15.5 to 30.0	3	100

[a] Not all detected oil components are listed, only the transformation of well-detected products which could be analyzed in extracts after alkaline extraction (pH 9) of MSM medium and crude oil (1 mL in 100-mL medium) are shown

Table 3 Aliphatic oil components detected by GC/MS and aliphatic acids formed during growth on crude oil by *Gordonia rubripertincta* SBUG 1971, *Gordonia rubripertincta* SBUG 1972 and *Rhodococcus* sp. SBUG 1968

Detected oil components Name	Corresponding acids formed Name[a]
n-Alkanes	
Nonane to dotriacontane	Heptanoic acid M1
Mono- and dimethyl- and monoethyl-substituted alkanes	
Monomethyl-substituted alkanes	2-Methyl-hexanoic acid M6
Dimethyl-substituted alkanes	2,3-Dimethyl-butanoic acid M7
Monoethyl-substituted alkanes	2-Ethyl-pentanoic acid M2
	2-Ethyl-hexanoic acid M3
	2-Ethyl-heptanoic acid M4
	2-Ethyl-octanoic acid M5
Alkylcyclohexanes	
Odd-numbered alkyl-substituted cyclohexanes	Cyclohexylpropanoic acid M8
	Cyclohexane-carboxylic acid M9
	1-Cyclohexene-1-carboxylic acid M10
	1,4-Cyclohexadiene-1-carboxylic acid M11
Even-numbered alkyl-substituted cyclohexanes	Cyclohexylacetic acid M12
Methyl-substituted *n*-alkylcyclohexanes	4-Methylcyclohexane-carboxylic acid M13
	1-Cyclohexene-1,2-dicarboxylic acid M14
Alkylcyclopentanes	
n-Alkylcyclopentanes	Cyclopentane-carboxylic acid M15
	1-Cyclopentene-1-carboxylic acid M16
Methyl-substituted *n*-alkylcyclopentanes	3-Methylcyclopentane-carboxylic acid M17
	3-Methyl-2-cyclopentene-1,2-dicarboxylic acid M18
	3-Methylenecyclopentane-1,2-dicarboxylic acid M19
Alkylbenzenes	
Odd-numbered alkyl-substituted benzenes	Phenylpropanoic acid M20
	Benzoic acid (phenyl-carboxylic acid) M21
	2-Hydroxybenzoic acid M22
	3,4-Dihydroxybenzoic acid M23
Even-numbered alkyl-substituted benzenes	Phenylacetic acid M24
Poly-*n*-alkyl-substituted benzenes	4-Methylbenzoic acid M25
	3-Methylbenzoic acid M26
	4-Ethylbenzoic acid M27
	2,4-Dimethylbenzoic acid M28
	2,3-Dimethylbenzoic acid M29
	2,4,5-Trimethylbenzoic acid M30
Branched-chain-alkyl-substituted benzenes	β-Methyl-phenylacetic acid M31
	γ-Methyl-γ-phenylpropanoic acid M32
	Methyl-β-methyl-phenylacetic acids M33, M34, M35
Alkylnaphthalenes	
Odd-numbered alkyl-substituted naphthalenes	1-Naphthylpropanoic acid M36
	1-Naphthalene-carboxylic acid M37
Even-numbered alkyl-substituted naphthalenes	1-Naphthylbutyric acid M38
	1-Naphtylacetic acid M39
	2-Naphthylacetic acid M40
Poly-*n*-alkyl-substituted naphthalenes	Methyl-naphthalene-carboxylic acid M41
Alkylbiphenyls	
Odd-numbered alkyl-substituted biphenyls	4-Biphenyl-carboxylic acid M42
Even-numbered alkyl-substituted biphenyls	4-Biphenylacetic acid M43
Poly-*n*-alkyl-substituted biphenyls	4,4′-Biphenyl-dicarboxylic acid M44

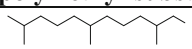
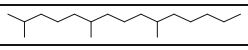
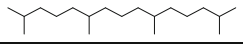
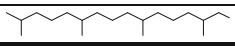
[a] See structures of the formed acids in Supplementary Material Tables S1, S2, S3, S4, S5, and S6

substituted alkanoic acids (M2–M7) were obtained. Besides the *n*-alkyl and branched chain alkyl carboxylic acids, seven cyclohexylalkanoic acids (M8–M14) and five cyclopentylalkanoic acids (M15–M19) were identified with cyclohexylacetic acid (M12) being present in the highest yield. The degradation of *n*-alkylbenzenes led to the formation of phenylpropionic acid (M20), benzoic acid (M21), and 2-hydroxybenzoic acid (M22) or 3,4-dihydroxybenzoic acid (M23) if odd-numbered *n*-alkylbenzenes were consumed and to phenylacetic acid (M24) in the case of even-numbered substrates. Furthermore, six different alkyl-substituted benzoic acids (M25–M30) were recovered from poly-*n*-alkyl-substituted benzenes. Additionally, five branched-chain-alkyl-substituted aromatic acids (M31–M35) were identified as transformation products. The transformation of *n*-alkylnaphthalenes led to the formation of 1-naphthylpropionic acid (M36) and 1-naphthalene-carboxylic acid (M37) from odd-numbered *n*-alkylnaphthalenes and to 1-naphthylbutyric acid (M38) and 1-naphthylacetic acid (M39) from even-numbered substrates. Two other substituted naphthylacetic (M40) and naphthalene-carboxylic (M41) acids were identified as transformation products. Similar transformations of alkylbiphenyls resulted in the biphenylalkanoic acids M42, M43, and M44.

Crude oil consumption of reference strains

In summary, *G. rubripertincta* SBUG 1971 and SBUG 1972 and *Rhodococcus* sp. SBUG 1968 were able to degrade or transform more than 80 different components of the crude oil. All these components contain at least one alkyl chain and were therefore transformed by alkane monooxygenases or by the very similar class of monooxygenases oxidizing alkane substituents of cycloaliphatic or aromatic rings. However, gordoniae and rhodococci are also able to directly oxidize aromatic rings either by ring-monooxygenases or ring-dioxygenases to form the corresponding epoxides or dihydrodiols and that are transformed to phenolic and catecholic compounds that are then further degraded (Andreoni et al. 2000; Arenskötter et al. 2004; de Carvalho and da Fonseca 2005b; Gallego et al. 2014; Kästner et al. 1994; Kim et al. 2002; Lin et al. 2012; Warhurst and Fewson 1994). The *R. erythropolis* SBUG 271 is able to hydroxylate and cleave the aromatic ring of biphenyl and dibenzofuran (Stope et al. 2002) and to hydroxylate and further transform carbazole, dibenzothiophene, and fluorene (Waldau et al. 2009). We therefore used *R. erythropolis* SBUG 271 as reference strain for direct attack at aromatic rings. This strain was incubated with crude oil, and whole cultures were extracted and analyzed as described for the isolated species. All the compounds named in Table 2 were handled by *R. erythropolis* SBUG 271 in the same way as described for the newly isolated strains with one exception.

Table 4 Degradation of *n*-alkanes and branched chain alkanes during growth of *Nocardia cyriacigeorgica* SBUG 1472 on crude oil

Oil components	Degradation [%]	
	7 days	*14 days*
***n*-alkanes**		
$C_{12}H_{26}$	100	100
$C_{13}H_{28}$	100	100
$C_{14}H_{30}$	100	100
$C_{15}H_{32}$	91	100
$C_{16}H_{34}$	89	100
$C_{17}H_{36}$	89	100
$C_{18}H_{38}$	87	100
$C_{19}H_{40}$	84	100
$C_{20}H_{42}$	79	100
$C_{21}H_{44}$	78	100
$C_{22}H_{46}$	78	100
$C_{23}H_{48}$	76	100
$C_{24}H_{50}$	76	100
$C_{25}H_{52}$	66	100
$C_{26}H_{54}$	65	100
$C_{27}H_{56}$	63	100
$C_{28}H_{58}$	61	100
$C_{29}H_{60}$	48	100
$C_{30}H_{62}$	51	100
$C_{31}H_{64}$	41	100
$C_{32}H_{66}$	41	100
polymethyl-substituted alkanes		
	0	23
	28	61
	0	47
	0	39

R. erythropolis SBUG 271, a biphenyl degrader, was able to consume biphenyl in crude oil completely (Supplementary Table S7 A) whereas naphthalene and phenanthrene remained untouched.

Furthermore, *G. rubripertincta* SBUG 1971, *G. rubripertincta* SBUG 1972, *Rhodococcus* sp. SBUG 1968, and also *R. erythropolis* SBUG 271 were not able to transform polymethyl-substituted alkanes, e.g., 2,6,10-trimethyldodecane, 2,6,10-trimethylpentadecane, pristane (2,6,10,14-tetramethylpentadecane), and 2,6,10,14-tetramethylhexadecane during growth on crude oil, whereas a *Rhodococcus* sp. strain (Komukai-Nakamura et al. 1996), later identified as *R. erythropolis*, was reported to degrade pristane. In order to determine if the pristane and other polymethyl-substituted alkanes of the Kazakh crude oil can in principle be degraded by pristane-degrading microorganisms and to compare these results with the results of the Kazakh strains, we used *N. cyriacigeorgica* SBUG 1472, a pristane degrader (Nhi-Cong et al. 2010), as

reference strain. This strain was also incubated, and whole cultures were extracted and analyzed as described for the isolated species. After 7 days of incubation with *N. cyriacigeorgica* SBUG 1472, the *n*-alkanes dodecane, tridecane, and tetradecane were completely degraded whereas *n*-alkanes with progressively longer chain lengths were progressively less degraded (Table 4). The polymethyl-substituted alkanes 2,6,10-trimethyldodecane, pristane (2,6,10,14-tetramethylpentadecane), and 2,6,10,14-tetramethylhexadecane were not transformed, whereas 2,6,10-trimethylpentadecane was degraded to a low degree. After 14 days, all *n*-alkanes were completely consumed by *N. cyriacigeorgica* and all the above named polymethyl-substituted alkanes were also utilized to a considerable extent (20–60 %).

Growth experiments on aromatic hydrocarbons

Growth of all bacteria was carried out on biphenyl and naphthalene in order to determine if the strains are able to degrade these substrates as pure compounds and to compare these results with their capacity to degrade biphenyl and naphthalene mixed into crude oil (Supplementary Material Table S7). None of the strains could grow on naphthalene, and only *R. erythropolis* SBUG 271 could use biphenyl as growth substrate. Furthermore, naphthalene in crude oil could not be transformed or degraded and biphenyl in crude oil could only be consumed by *R. erythropolis* SBUG 271.

Influence of microbial inoculation of barley seeds on the plant development

Barley seeds were inoculated with the isolated bacterial strains *G. rubripertincta* SBUG 1971, *G. rubripertincta* SBUG 1972, and *Rhodococcus* sp. SBUG 1968 and sown in oil-containing sand. Plants were grown for 10 days and then the lengths of shoots and roots were measured (Fig. 1). The best protective effect on plants was observed in systems inoculated with *G. rubripertincta* SBUG 1971 and SBUG 1972. This treatment of seeds stimulated the growth of barley seedlings, increasing the growth of shoots by 86 and 42 % and of roots by 42 and 78 %, respectively, compared with their growth in oil-containing sand without inoculation. In the case of *Rhodococcus* sp. SBUG 1968, weak effects on the root system were noted, but the growth of shoots was increased by 100 %. In parallel, experiments with inoculation of barley seeds with bacteria and their growth in sand without oil were conducted. The inoculation of seeds with bacteria had no significant effect on growth of barley. Only inoculation by *Rhodococcus* sp. SBUG 1968 marginally increased the length of shoot and root.

Discussion

Three oil-degrading bacterial strains have been isolated from the rhizosphere and characterized in respect to their ability to use different types of oil as the sole carbon and energy source. These microorganisms were identified as *G. rubripertincta* SBUG 1971, *G. rubripertincta* SBUG 1972, and *Rhodococcus* sp. SBUG 1968. Few *R. erythropolis* strains were also isolated from the rhizosphere (Cirou et al. 2007; Jussila et al. 2006). Rhodococci have been described as major components of the bacterial population of the microenvironment of potato rhizosphere (Diallo et al. 2011). In contrast, the isolation of gordoniae from the rhizosphere is only known from mangrove rhizosphere (Arenskötter et al. 2004; Takeuchi and Hatano 1998). Hydrocarbon utilizing gordoniae have also been isolated from oil-contaminated soils (Kummer et al. 1999; Nicdao and Rivera 2012). Altogether, gordoniae have been found relatively seldom in the rhizosphere but often in bulk soil; therefore, it is unclear if

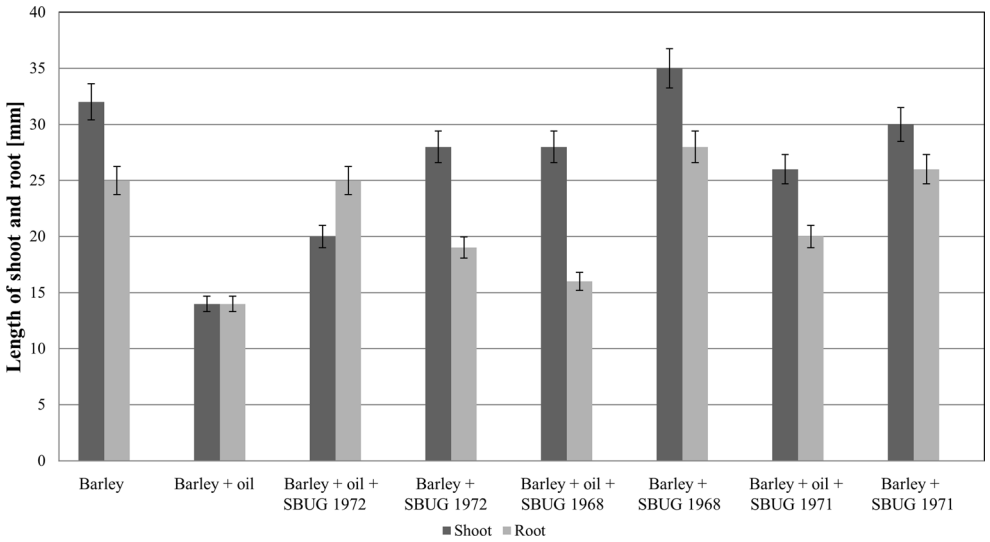


Fig. 1 Influence of microbial inoculation of barley seeds on the plant development in oil-containing sand. *G. rubripertincta* SBUG 1971 (SBUG 1971), *G. rubripertincta* SBUG 1972 (SBUG 1972), and *Rhodococcus* sp. SBUG 1968 (SBUG 1968)

our isolated species can unambiguously be identified as rhizosphere microorganisms.

To study the spectrum of hydrocarbons utilized by our isolated strains, we used low viscosity crude oil with a low content of resins and asphaltenes, and all three strains showed good to excellent growth on the sixth and on the tenth day of incubation. However, diesel fuel, fuel oil, and gasoline have a more viscous structure due to their higher content of sulfur, resins, asphaltenes, and other components and are consequently more difficult to degrade (Gailiūtė et al. 2011; Khorasani et al. 2013). In line with this, *Rhodococcus* sp. SBUG 1968 grew poorly on medium with diesel fuel, fuel oil, or gasoline by the sixth day of cultivation and *G. rubripertincta* SBUG 1972 grew poorly on medium with fuel oil by the sixth day. Hydrocarbons utilizing gordoniae and rhodococci have been previously isolated from oil-contaminated sludge and soil (Kummer et al. 1999; Nicdao and Rivera 2012; Song et al. 2011; Young et al. 2005). They have been reported to be able to grow on diesel-containing medium or on a wide range of saturated hydrocarbons and/or polyaromatics.

We show that our isolates can biodegrade an unusually wide spectrum of hydrocarbons as substrates (more than 80 oil components; Table 2). The first important result to be noticed is that all three isolates can transform mainly alkyl chains of components of the crude oil. *n*-Alkanes with chains ranging from 12 to 32 carbon atoms and monomethyl- and monoethyl-substituted alkanes (C_{12}–C_{23}) were efficiently used as substrates. Gordoniae have been reported to degrade alkanes in the range of C_{12} to C_{36} (Kummer et al. 1999; Lo Piccolo et al. 2011; Nicdao and Rivera 2012) and rhodococci can utilize a broad range of branched and *n*-alkanes with chain length of C_5 to C_{35} (de Carvalho and da Fonseca 2005a; de Carvalho et al. 2005; de Carvalho et al. 2009; Komukai-Nakamura et al. 1996; Rapp and Gabriel-Jürgens 2003; Whyte et al. 1998). The monoterminal degradation pathway for *n*-alkanes and branched chain alkanes involves primary hydroxylation at the terminal methyl group by alkane 1-monooxygenases (Shen et al. 2010), further oxidation to monocarboxylic acids and ß-oxidation forming various alkanoic acids with different chain length as intermediates (McKenna and Kallio 1971; Rojo 2009; Singh et al. 2012; van Beilen et al. 2003; Watkinson and Morgan 1990; Widdel and Musat 2010). This explains the recovery of *n*-heptanoic acid (M1), four monoethyl-substituted and two methyl-substituted alkanoic acids (M2–M7) from *n*-alkane and methyl- and ethyl-substituted alkane transformation by the new isolates (Table 3). The accumulated intermediate *n*-heptanoic acid (M1) is only one of the intermediates on the pathway to acetyl CoA and propionyl CoA (Fig. 2a) starting from *n*-alkanes as substrates.

Using alkylcyclohexanes and alkylcyclopentanes and alkyl-substituted benzenes, naphthalenes and biphenyls of the Kazakh crude oil as substrates 7 cyclohexylalkanoic and 5 cyclopentylalkanoic acids as well as 16 phenyl, 6 naphthyl, and 3 biphenyl alkanoic acids were identified as metabolites during transformation by the three isolates (Table 3; Fig. 2b, c). The most common pathway of oxidation of alkyl chains is the monoterminal oxidation via an alcohol and an aldehyde intermediate (Morgan and Watkinson 1994; Ratledge 1978) to the corresponding carboxylic acid. This pathway of microbial transformation has been found for alkyl substituents of cyclohexane (Beam and Perry 1974; Feinberg et al. 1980; Perry 1977; Solano-Serena et al. 2008), heteroaromatics, and aromatics (Awe et al. 2008; Fedorak et al. 1996; Herter et al. 2012; Mikolasch et al. 2003; Poole and Whitaker 1997; Webley et al. 1956; White and Russell 1994). Both carboxylic acid and also acetic acid derivatives were identified from even-numbered and also from odd-numbered *n*-alkyl substituents (Awe et al. 2008; Beam and Perry 1974; Dutta and Harayama 2001; Fedorak et al. 1988; Fedorak and Westlake 1986; Herter et al. 2012; Koma et al. 2005; Rontani and Bonin 1992; Sariasla et al. 1974). Therefore, it is not entirely clear, whether the carboxylic and acetic acid derivatives derived from even- or from odd-numbered *n*-alkyl substituted parent compounds if mixtures like crude oil are used as substrates.

On the other hand, degradation of alkylbenzenes by bacteria can also start with an attack on the aromatic ring by oxygenation or simultaneously on both the alkyl side chain by monooxygenation and on the aromatic ring by monooxygenation or dioxygenation (Awe et al. 2009; Bhatia and Singh 1996; Jigami et al. 1979; Smith and Ratledge 1989). We found two ring attacked products—the 2-hydroxybenzoic acid (M22) and the 3,4-dihydroxybenzoic acid (M23)—in the incubation experiments. These are two important intermediates on the pathway of complete degradation of *n*-alkyl substituted benzenes, but we had no evidence of direct oxidation of aromatic rings either by ring-monooxygenases or ring-dioxygenases before alkyl chain metabolism. Furthermore, naphthalene, biphenyl, and phenanthrene of crude oil were not degraded by these strains, although the degradation of aromatic compounds by gordoniae and rhodococci is well established (Andreoni et al. 2000; Arenskötter et al. 2004; de Carvalho and da Fonseca 2005b; Gallego et al. 2014; Kästner et al. 1994; Kim et al. 2002; Lin et al. 2012; Warhurst and Fewson 1994). *R. erythropolis* SBUG 271 was able to degrade biphenyl of the Kazakh crude oil as expected for a well-known biphenyl degrader (Stope et al. 2002; Waldau et al. 2009). However, naphthalene and phenanthrene were not transformed in the crude oil or when supplied as a pure substrate, although this strain is described to transform the aromatic rings of dibenzofuran (Stope et al. 2002), carbazole, dibenzothiophene, and fluorene (Waldau et al. 2009). All these aromatics were degraded via cometabolic biotransformation or after preculture with biphenyl and that may explain why they were not degraded in complex mixtures like crude oil during growth without preculture. Our new isolates were not able to grow on biphenyl and naphthalene or to transform these substrates in crude oil. This suggests that these strains might be used in mixed

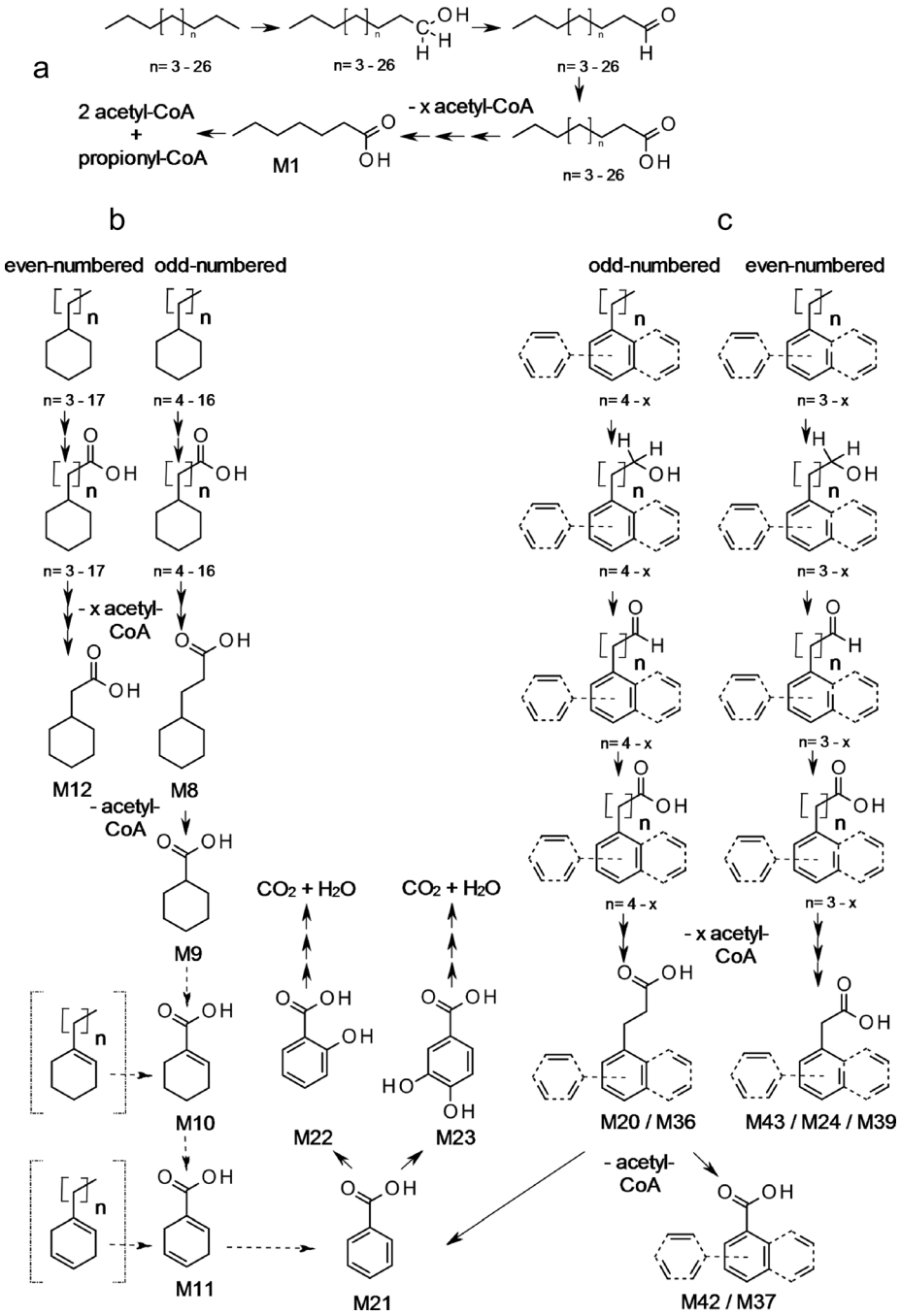


Fig. 2 Transformation pathways **a** of *n*-alkanes; **b** of *n*-alkyl-substituted cyclohexanes; and **c** of *n*-alkyl-substituted benzenes, naphthalenes, and biphenyls with identified metabolites (M1–M44)

populations with, e.g., biphenyl and polyaromatic hydrocarbon degraders for further experiments.

Cyclohexylacetic acid (M12) was formed by *G. rubripertincta* SBUG 1971 and SBUG 1972 in higher yield than any of the other acids. Feinberg et al. (1980) reported the further degradation of cyclohexylacetic acid by use of a mixed culture of *Mycobacterium* and *Arthrobacter* species indicating the potential of mixed populations for bioremediation of special oil components. The cyclohexane-carboxylic acid (M9) and cyclohexylacetic acid (M12) can be further transformed via different pathways. On one hand side, M9 is metabolized to 1-cyclohexene-1-carboxylic acid (M10) and pimelic acid by continued oxidation (Blakley and Papish 1982; Rontani and Bonin 1992; Solano-Serena et al. 2008). The transformation of M9 to 4-hydroxybenzoic acid via 4-oxocyclohexanecarboxylic acid has been described in some bacteria (Blakley and Papish 1982; Smith and Callely 1975). In contrast, the formation of benzoic acid (M21) from M9 via 1-cyclohexene-1-carboxylic acid (M10) was shown by Dutta and Harayama (2001) and Koma et al. (2005). We have shown the occurrence of M9, M10, and 1,4-cyclohexadiene-1-carboxylic acid (M11) and also benzoic acid (M21), which can also be

obtained from the degradation of alkyl-substituted benzenes (Fig. 2b, c). This indicates that the transformation of different components of crude oil is based on convergent degradation pathways. To the best of our knowledge, this is the first report of 1,4-cyclohexadiene-1-carboxylic acid (M11) as a metabolite in the degradation pathway of odd-numbered alkyl-substituted cycloalkane oil components. Although we did not detect any *n*-alkylcyclohexenes in the crude oil, it is possible that some of these substances are present in traces so that 1-cyclohexene-1-carboxylic acid (M10) and 1,4-cyclohexadiene-1-carboxylic acid (M11) could also be derived from such substances. However, in that case, we should have also detected 1-cyclohexene-1-acetic and 1,4-cyclohexadiene-1-acetic acid, but neither of these compounds were formed.

n-Alkyl-methylcyclohexanes and methylcyclopentanes have been transformed by our new isolates to the products 4-methylcyclohexane-carboxylic acid (M13), 1-cyclohexene-1,2-dicarboxylic acid (M14), 3-methylcyclopentane-carboxylic acid (M17), 3-methyl-2-cyclopentene-1,2-dicarboxylic acid (M18), and 3-methylenecyclopentane-1,2-dicarboxylic acid (M19). This is the first report of the production of 1-cycloalkene-1,2-dicarboxylic acids as metabolites. These cycloalkene-1,2-dicarboxylic acids can be formed from poly-*n*-alkylcycloalkanes by hydroxylation and further oxidation of two *n*-alkyl chains in the same manner as described above.

All of the four monoethyl-substituted and two methyl-substituted alkanoic acids (M2–M7) contain the branching point in the α-position to the carboxylic group. From these results—together with the persistence of the polyalkyl-substituted alkanes 2,6,10-trimethyldodecane, 2,6,10-trimethylpentadecane, pristane (2,6,10,14-tetramethylpentadecane), and 2,6,10,14-tetramethylhexadecane—we conclude that our microorganisms are not able to degrade branched chain alkanes completely. They can hydroxylate the terminal methyl group, oxidize the resulting alcohol and the aldehyde, and ß-oxidize the resulting acid up to the branching point. Only a few bacteria such as *Rhodococcus ruber*, *Mycobacterium neoaurum*, and *N. cyriacigeorgica* are able to mineralize and utilize branched chain hydrocarbons completely (Nhi-Cong et al. 2009; Nhi-Cong et al. 2010). As expected, *N. cyriacigeorgica* SBUG 1472 degraded the polymethyl-substituted alkanes of the Kazakh crude oil, but not before all *n*-alkanes have been completely consumed (Table 4). This suggests that the degradation of branched chain alkanes is repressed by the consumption of *n*-alkanes by *N. cyriacigeorgica* SBUG 1472. Furthermore, it indicates that our newly isolated strains and *R. erythropolis* SBUG 271 are not able to degrade polymethyl-substituted alkanes because these compounds remained untouched after the total degradation of *n*-alkanes. Thus, the newly isolated strains should also be used in mixed populations with branched chain alkane degraders for further experiments.

On the basis of all transformation results, we conclude that the main components of the Kazakh crude oil can be degraded by *G. rubripertincta* SBUG 1971, *G. rubripertincta* SBUG 1972, and *Rhodococcus* sp. SBUG 1968. Furthermore, these bacteria isolated from the rhizosphere helped to improve the growth of barley seeds in the presence of crude oil due to detoxification of oil constituents. For rhizoremediation, an important contribution to the degradation of pollutants is attributed to microorganisms (Graj et al. 2013; Kuiper et al. 2004), suggesting that the newly isolated species might be used for rhizoremediation projects in Kazakhstan and elsewhere.

Acknowledgments We thank R. Jack (Institute of Immunology, University of Greifswald) for help in preparing the manuscript. We thank Central Asian Biodiversity Network (CABNET), in particular the project manager Michael Manthey (Institute of Botany and Landscape Ecology, University of Greifswald), for the opportunity to establish active contacts between scientists from the Al-Farabi Kazakh National University and the University of Greifswald. Financial support from Deutscher Akademischer Austauschdienst (DAAD) (project code 50754935, project title "CABNET-Central Asian Biodiversity Network") is gratefully acknowledged.

References

Andreoni V, Bernasconi S, Colombo M, van Beilen JB, Cavalca L (2000) Detection of genes for alkane and naphthalene catabolism in *Rhodococcus* sp. strain 1BN. Environ Microbiol 2(5):572–577. doi:10.1046/j.1462-2920.2000.00134.x

Arenskötter M, Bröker D, Steinbüchel A (2004) Biology of the metabolically diverse genus *Gordonia*. Appl Environ Microbiol 70(6): 3195–3204. doi:10.1128/aem. 70.6.3195-3204.2004

Atlas RM (1991) Microbial hydrocarbon degradation - bioremediation of oil spills. J Chem Technol Biotechnol 52(2):149–156

Atlas RM, Atlas MC (1991) Biodegradation of oil and bioremediation of oil spills. Curr Opin Biotechnol 2(3):440–443. doi:10.1016/s0958-1669(05)80153-3

Awe S, Mikolasch A, Hammer E, Schauer F (2008) Degradation of phenylalkanes and characterization of aromatic intermediates acting as growth inhibiting substances in hydrocarbon utilizing yeast *Candida maltosa*. Int Biodeterior Biodegrad 62(4):408–414. doi: 10.1016/j.ibiod.2008.03.007

Awe S, Mikolasch A, Schauer F (2009) Formation of coumarines during the degradation of alkyl substituted aromatic oil components by the yeast *Trichosporon asahii*. Appl Microbiol Biotechnol 84(5):965–976. doi:10.1007/s00253-009-2044-2

Beam HW, Perry JJ (1974) Microbial degradation and assimilation of *n*-alkyl-substituted cycloparaffins. J Bacteriol 118(2):394–399

Bhatia M, Singh HD (1996) Biodegradation of commercial linear alkyl benzenes by *Nocardia amarae*. J Biosci 21(4):487–496. doi:10.1007/bf02703213

Blakley ER, Papish B (1982) The metabolism of cyclohexanecarboxylic acid and 3-cyclohexenecarboxylic acid by *Pseudomonas putida*. Can J Microbiol 28(12):1324–1329

Cirou A, Diallo S, Kurt C, Latour X, Faure D (2007) Growth promotion of quorum-quenching bacteria in the rhizosphere of *Solanum tuberosum*. Environ Microbiol 9(6):1511–1522. doi:10.1111/j.1462-2920.2007.01270.x

Clayton RA, Sutton G, Hinkle PS, Bult C, Fields C (1995) Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. Int J Syst Bacteriol 45(3):595–599

De Boer TD, Backer HJ (1956) Diazomethane. In: J LN (ed) Organic synthesis, vol 36. Wiley, New York, NY, pp 14–16

de Carvalho CCCR, da Fonseca MMR (2005a) Degradation of hydrocarbons and alcohols at different temperatures and salinities by *Rhodococcus erythropolis* DCL14. FEMS Microbiol Ecol 51(3): 389–399. doi:10.1016/j.femsec.2004.09.010

de Carvalho CCCR, da Fonseca MMR (2005b) The remarkable *Rhodococcus erythropolis*. Appl Microbiol Biotechnol 67(6):715–726. doi:10.1007/s00253-005-1932-3

de Carvalho CCCR, Parreno-Marchante B, Neumann G, da Fonseca MMR, Heipieper HJ (2005) Adaptation of *Rhodococcus erythropolis* DCL14 to growth on *n*-alkanes, alcohols and terpenes. Appl Microbiol Biotechnol 67(3):383–388. doi:10.1007/s00253-004-1750-z

de Carvalho CCCR, Wick LY, Heipieper HJ (2009) Cell wall adaptations of planktonic and biofilm *Rhodococcus erythropolis* cells to growth on C5 to C16 *n*-alkane hydrocarbons. Appl Microbiol Biotechnol 82(2):311–320. doi:10.1007/s00253-008-1809-3

Diallo S, Crepin A, Barbey C, Orange N, Burini J-F, Latour X (2011) Mechanisms and recent advances in biological control mediated through the potato rhizosphere. FEMS Microbiol Ecol 75(3):351–364. doi:10.1111/j.1574-6941.2010.01023.x

Dua M, Singh A, Sethunathan N, Johri AK (2002) Biotechnology and bioremediation: successes and limitations. Appl Microbiol Biotechnol 59(2–3):143–152. doi:10.1007/s00253-002-1024-6

Dutta TK, Harayama S (2001) Biodegradation of *n*-alkylcycloalkanes and *n*-alkylbenzenes via new pathways in *Alcanivorax* sp. strain MBIC 4326. Appl Environ Microbiol 67(4):1970–1974. doi:10.1128/aem. 67.4.1970-1974.2001

Fedorak PM, Westlake DWS (1986) Fungal metabolism of *n*-alkylbenzenes. Appl Environ Microbiol 51(2):435–437

Fedorak PM, Payzant JD, Montgomery DS, Westlake DWS (1988) Microbial degradation of *n*-alkyl tetrahydrothiophenes found in petroleum. Appl Environ Microbiol 54(5):1243–1248

Fedorak PM, Coy DL, Peakman TM (1996) Microbial metabolism of some 2,5-substituted thiophenes. Biodegradation 7(4):313–327. doi:10.1007/bf00115745

Feinberg EL, Ramage PIN, Trudgill PW (1980) The degradation of *n*-alkylcycloalkanes by a mixed bacterial culture. J Gen Microbiol 121(DEC):507–511

Gailiūtė I, Kavaliauskė M, Aikaitė-Stanaitienė J (2011) Changes in total oil hydrocarbon composition during degradation with sorbent bacterial preparation. Biologija 57:70–77

Gallego S, Vila J, Tauler M, Nieto JM, Breugelmans P, Springael D, Grifoll M (2014) Community structure and PAH ring-hydroxylating dioxygenase genes of a marine pyrene-degrading microbial consortium. Biodegradation 25(4):543–556. doi:10.1007/s10532-013-9680-z

Gerhardt KE, Huang X-D, Glick BR, Greenberg BM (2009) Phytoremediation and rhizoremediation of organic soil contaminants: potential and challenges. Plant Sci 176(1):20–30. doi:10.1016/j.plantsci.2008.09.014

Graj W, Lisiecki P, Szulc A, Chrzanowski L, Wojtera-Kwiczor J (2013) Bioaugmentation with petroleum-degrading consortia has a selective growth-promoting impact on crop plants germinated in diesel oil-contaminated soil. Water Air Soil Pollut 224:1676. doi:10.1007/s11270-013-1676-0

Heipieper HJ (2007) Bioremediation of soils contaminated with aromatic compounds: Effects of rhizosphere, bioavailability, gene regulation and stress adaptation. In: Heipieper HJ (ed) Bioremediation of soils contaminated with aromatic compounds. NATO Science Series IV-Earth and Environmental Sciences, vol 76, pp 1–4

Herter S, Mikolasch A, Schauer F (2012) Identification of phenylalkane derivatives when *Mycobacterium neoaurum* and *Rhodococcus erythropolis* were cultured in the presence of various phenylalkanes. Appl Microbiol Biotechnol 93(1):343–355. doi:10.1007/s00253-011-3415-z

Hong SH, Ryu H, Kim J, Cho KS (2011) Rhizoremediation of diesel-contaminated soil using the plant growth-promoting rhizobacterium *Gordonia* sp. S2RP-17. Biodegradation 22(3):593–601. doi:10.1007/s10532-010-9432-2

Hundt K, Wagner M, Becher D, Hammer E, Schauer F (1998) Effect of selected environmental factors on degradation and mineralization of biaryl compounds by the bacterium *Ralstonia pickettii* in soil and compost. Chemosphere 36(10):2321–2335

Jigami Y, Kawasaki Y, Omori T, Minoda Y (1979) Coexistence of different pathways in the metabolism of *n*-propylbenzene by *Pseudomonas* sp. Appl Environ Microbiol 38(5):783–788

Jussila MM, Jurgens G, Lindstrom K, Suominen L (2006) Genetic diversity of culturable bacteria in oil-contaminated rhizosphere of *Galega orientalis*. Environ Pollut 139(2):244–257. doi:10.1016/j.envpol.2005.05.013

Kästner M, Breuer-Jammali M, Mahro B (1994) Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). Appl Microbiol Biotechnol 41(2):267–273

Khorasani AC, Mashreghi M, Yaghmaei S (2013) Study on biodegradation of Mazut by newly isolated strain *Enterobacter cloacae* BBRC10061: improving and kinetic investigation. Iran J Environ Health Sci Eng 10(1):2–9. doi:10.1186/1735-2746-10-2

Kim D, Kim YS, Kim SK, Kim SW, Zylstra GJ, Kim YM, Kim E (2002) Monocyclic aromatic hydrocarbon degradation by *Rhodococcus* sp. strain DK17. Appl Environ Microbiol 68(7):3270–3278. doi:10.1128/aem. 68.7.3270-3278.2002

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721. doi:10.1099/ijs. 0.038075-0

Koma D, Sakashita Y, Kubota K, Fujii Y, Hasumi F, Chung SY, Kubo M (2005) Degradation pathways of cyclic alkanes in *Rhodococcus* sp NDKK48. Appl Microbiol Biotechnol 66(1):92–99. doi:10.1007/s00253-004-1623-5

Komukai-Nakamura S, Sugiura K, Yamauchi-Inomata Y, Toki H, Venkateswaran K, Yamamoto S, Tanaka H, Harayama S (1996) Construction of bacterial consortia that degrade Arabian light crude oil. J Ferment Bioeng 82(6):570–574. doi:10.1016/s0922-338x(97)81254-8

Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJ (2004) Rhizoremediation: a beneficial plant-microbe interaction. Mol Plant Microbe Interact 17(1):6–15. doi:10.1094/MPMI.2004.17.1.6

Kummer C, Schumann P, Stackebrandt E (1999) *Gordonia alkanivorans* sp. nov., isolated from tar-contaminated soil. Int J Syst Bacteriol 49 Pt 4:1513-22

Lin C-L, Shen F-T, Tan C-C, Huang C-C, Chen B-Y, Arun AB, Young C-C (2012) Characterization of *Gordonia* sp. strain CC-NAPH129-6 capable of naphthalene degradation. Microbiol Res 167(7):395–404. doi:10.1016/j.micres.2011.12.002

Lo Piccolo L, De Pasquale C, Fodale R, Puglia AM, Quatrini P (2011) Involvement of an alkane hydroxylase system of *Gordonia* sp strain SoCg in degradation of solid *n*-alkanes. Appl Environ Microbiol 77(4):1204–1213. doi:10.1128/aem. 02180-10

Maila MP, Randima P, Cloete TE (2005) Multispecies and monoculture rhizoremediation of polycyclic aromatic hydrocarbons (PAHs) from the soil. Int J Phytoremediat 7(2):87–98. doi:10.1080/16226510590950397

McKenna EJ, Kallio RE (1971) Microbial metabolism of the isoprenoid alkane pristane. Proc Natl Acad Sci U S A 68(7):1552–1554

Mikolasch A, Hammer E, Schauer F (2003) Synthesis of imidazol-2-yl amino acids by using cells from alkane-oxidizing bacteria. Appl Environ Microbiol 69(3):1670–1679. doi:10.1128/aem. 69.3.1670-1679.2003

Morgan P, Watkinson RJ (1994) Biodegradation of components of petroleum. In: Ratledge C (ed) Biochemistry of microbial degradation. Kluwer, Dordrecht, pp 1–31

Nhi-Cong LT, Mikolasch A, Klenk H-P, Schauer F (2009) Degradation of the multiple branched alkane 2,6,10,14-tetramethyl-pentadecane (pristane)

in *Rhodococcus ruber* and *Mycobacterium neoaurum*. Int Biodeterior Biodegrad 63(2):201–207. doi:10.1016/j.ibiod.2008.09.002

Nhi-Cong LT, Mikolasch A, Awe S, Sheikhany H, Klenk H-P, Schauer F (2010) Oxidation of aliphatic, branched chain, and aromatic hydrocarbons by *Nocardia cyriacigeorgica* isolated from oil-polluted sand samples collected in the Saudi Arabian Desert. J Basic Microbiol 50(3):241–253. doi:10.1002/jobm.200900358

Nicdao MAC, Rivera WL (2012) Two strains of *Gordonia terrae* isolated from used engine oil contaminated soil utilize short- to long-chain *n*-alkanes. Phil Sci Lett 5(2):199–208

Owsianiak M, Szulc A, Chrzanowski L, Cyplik P, Bogacki M, Olejnik-Schmidt AK, Heipieper HJ (2009) Biodegradation and surfactant-mediated biodegradation of diesel fuel by 218 microbial consortia are not correlated to cell surface hydrophobicity. Appl Microbiol Biotechnol 84(3):545–553. doi:10.1007/s00253-009-2040-6

Perry JJ (1977) Microbial metabolism of cyclic hydrocarbons and related compounds. Crit Rev Microbiol 5(4):387–412. doi:10.3109/10408417709102811

Poole PR, Whitaker G (1997) Biotransformation of 6-pentyl-2-pyrone by *Botrytis cinerea* in liquid cultures. J Agric Food Chem 45(1):249–252. doi:10.1021/jf9603644

Rapp P, Gabriel-Jürgens LH (2003) Degradation of alkanes and highly chlorinated benzenes, and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. and genetic characterization of its chlorobenzene dioxygenase. Microbiology 149(Pt 10):2879–2890

Ratledge C (1978) Degradation of aliphatic hydrocarbons. In: Watkinson RJ (ed) Developments in biodegradation of hydrocarbons, vol 1. Applied Science, London, pp 1–46

Rojo F (2009) Degradation of alkanes by bacteria. Environ Microbiol 11(10):2477–2490. doi:10.1111/j.1462-2920.2009.01948.x

Rontani JF, Bonin P (1992) Utilization of *n*-alkyl-substituted cyclohexanes by a marine *Alcaligenes*. Chemosphere 24(10):1441–1446. doi:10.1016/0045-6535(92)90266-t

Sariasla FS, Harper DB, Higgins IJ (1974) Microbial degradation of hydrocarbons catabolism of 1-phenyl-alkanes by *Nocardia salmonicolor*. Biochem J 140(1):31–45

Schumann P, Maier T (2014) MALDI-TOF mass spectrometry applied to classification and identification of bacteria. Method Microbiol. doi:10.1016/bs.mim.2014.06.002

Shen F-T, Young L-S, Hsieh M-F, Lin S-Y, Young C-C (2010) Molecular detection and phylogenetic analysis of the alkane 1-monooxygenase gene from *Gordonia* spp. Syst Appl Microbiol 33(2):53–59. doi:10.1016/j.syapm.2009.11.003

Singh SN, Kumari B, Mishra S (2012) Microbial degradation of alkanes. In: Singh SN (ed) Microbial degradation of xenobiotics, environmental science and engineering. Springer-Verlag, Berlin, Heidelberg, pp 439–469

Smith DI, Callely AG (1975) The microbial degradation of cyclohexane carboxylic acid. J Gen Microbiol 91(NOV):210–212

Smith MR, Ratledge C (1989) Catabolism of alkylbenzenes by *Pseudomonas* sp. NCIB 10643. Appl Microbiol Biotechnol 32(1):68–75

Solano-Serena F, Marchal R, Vandecasteele J-P (2008) Biodegradation of aliphatic and alicyclic hydrocarbons. In: Vandecasteele J-P (ed) Petroleum microbiology: concepts, environmental implications, industrial applications, vol 1. Editions Technip, Paris, pp 173–240

Soleimani M, Afyuni M, Hajabbasi MA, Nourbakhsh F, Sabzalian MR, Christensen JH (2010) Phytoremediation of an aged petroleum contaminated soil using endophyte infected and non-infected grasses. Chemosphere 81(9):1084–1090. doi:10.1016/j.chemosphere.2010.09.034

Song HG, Wang XP, Bartha R (1990) Bioremediation potential of terrestrial fuel spills. Appl Environ Microbiol 56(3):652–656

Song X, Xu Y, Li G, Zhang Y, Huang T, Hu Z (2011) Isolation, characterization of *Rhodococcus* sp. P14 capable of degrading high-molecular-weight polycyclic aromatic hydrocarbons and aliphatic hydrocarbons. Mar Pollut Bull 62(10):2122–2128. doi:10.1016/j.marpolbul.2011.07.013

Stead DE, Sellwood JE, Wilson J, Viney I (1992) Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. J Appl Bacteriol 72:315–321. doi:10.1111/j.1365-2672.1992.tb01841.x

Stope MB, Becher D, Hammer E, Schauer F (2002) Cometabolic ring fission of dibenzofuran by Gram-negative and Gram-positive biphenyl-utilizing bacteria. Appl Microbiol Biotechnol 59(1):62–67. doi:10.1007/s00253-0002-0979-7

Suslow TV, Schroth MN, Isaka M (1982) Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology 72:917–918. doi:10.1094/Phyto-77-917

Takeuchi M, Hatano K (1998) *Gordonia rhizosphera* sp. nov, isolated from the mangrove rhizosphere. Int J Syst Bacteriol 48:907–912

Táncsics A, Benedek T, Farkas M, Máthé I, Márialigeti K, Szoboszlay S, Kukolya J, Kriszt B (2014) Sequence analysis of 16S rRNA, gyrB and catA genes and DNA–DNA hybridization reveal that *Rhodococcus jialingiae* is a later synonym of *Rhodococcus qingshengii*. Int J Syst Evol Microbiol 64:298–301

Tjessem K, Aaberg A (1983) Photochemical transformation and degradation of petroleum residues in the marine environment. Chemosphere 12(11–1):1373–1394. doi:10.1016/0045-6535(83)90070-x

Upadhyay SK, Singh DP, Saikia R (2009) Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of wheat under saline condition. Curr Microbiol 59(5):489–496. doi:10.1007/s00284-009-9464-1

van Beilen JB, Li Z, Duetz WA, Smits THM, Witholt B (2003) Diversity of alkane hydroxylase systems in the environment. Oil Gas Sci Technol 58(4):427–440. doi:10.2516/ogst:2003026

Waldau D, Methling K, Mikolasch A, Schauer F (2009) Characterization of new oxidation products of 9H-carbazole and structure related compounds by biphenyl-utilizing bacteria. Appl Microbiol Biotechnol 81(6):1023–1031. doi:10.1007/s00253-008-1723-8

Wang XP, Bartha R (1990) Effects of bioremediation on residues, activity and toxicity in soil contaminated by fuel spills. Soil Biol Biochem 22(4):501–505. doi:10.1016/0038-0717(90)90185-3

Warhurst AM, Fewson CA (1994) Biotransformations catalyzed by the genus *Rhodococcus*. Crit Rev Biotechnol 14(1):29–73. doi:10.3109/07388559409079833

Watkinson RJ, Morgan P (1990) Physiology of aliphatic hydrocarbon-degrading microorganisms. Biodegradation 1(2–3):79–92

Webley DM, Duff RB, Farmer VC (1956) Evidence for β-oxidation in the metabolism of saturated aliphatic hydrocarbons by soil species of *Nocardia*. Nature 178(4548):1467–1468. doi:10.1038/1781467b0

Werle E, Schneider C, Renner M, Volker M, Fiehn W (1994) Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Res 22(20):4354–4355

White GF, Russell NJ (1994) Biodegradation of anionic surfactants and related molecules. In: Ratledge C (ed) Biochemistry of microbial degradation. Kluwer, Dordrecht, pp 143–177

Whyte LG, Hawari J, Zhou E, Bourbonniere L, Inniss WE, Greer CW (1998) Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. Appl Environ Microbiol 64(7):2578–2584

Widdel F, Musat F (2010) Diversity and common principles in enzymatic activation of hydrocarbons. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, Heidelberg, pp 981–1009

Young CC, Lin TC, Yeh MS, Shen FT, Chang JS (2005) Identification and kinetic characteristics of an indigenous diesel-degrading *Gordonia alkanivorans* strain. World J Microbiol Biotechnol 21(8–9):1409–1414. doi:10.1007/s11274-005-5742-7