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Biodegradation and conversion of alkanes and crude oil by a marine *Rhodococcus* sp.

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Abstract

A hydrocarbon degrader isolated from a chronically oil-polluted marine site was identified as *Rhodococcus* sp. on the basis of morphology, fatty acid methyl ester pattern, cell wall analysis, biochemical tests and G + C content of DNA. It degraded up to 50% of the aliphatic fraction of Assam crude oil, in seawater supplemented with 35 mM nitrogen as urea and 0.1 mM phosphorus as dipotassium hydrogen orthophosphate, after 72 h at 30 °C and 150 revolutions per minute. The relative percentage of intracellular fatty acid was higher in hydrocarbon-grown cells compared to fructose-grown cells. The fatty acids C_{16}, $C_{16:1}$, C_{18} and $C_{18:1}$ were constitutively present regardless of the growth substrate. In addition to these constitutive acids, other intracellular fatty acids varied in correlation to the hydrocarbon chain length supplied as a substrate. When grown on odd carbon number alkanes, the isolate released only monocarboxylic acids into the growth medium. On even carbon number alkanes only dicarboxylic acids were produced.

Introduction

Crude oil transport, industrial run-offs and tanker accidents are responsible for chronic pollution of estuarine and marine ecosystems by oil and hydrocarbons. In such environments a local flora capable of degrading crude oil is known to establish itself (Bobra et al. 1980). Among these the *Nocardia* group of actinomycetes are important (Lacey 1988) and *Rhodococcus* strains are known to be dominant in such ecosystems and efficient in crude oil degradation (Sorkhoh et al. 1990; Bredholt et al. 1998). Warhurst & Fewson (1994) in their review of the rhodococci have shown that this genus is capable of biotransformation of a large variety of chemicals including alkanes.

Alkane degradation by microorganisms occurs by monoterminal, diterminal and subterminal pathways. Organisms of the *Rhodococcus* group have been reported to possess either monoterminal (Warhurst & Fewson 1994), diterminal (Broadway et al. 1993) or only subterminal pathway (Ludwig et al. 1995). Occurrence of both terminal and subterminal modes of catabolism of alkanes have also been reported (Woods & Murrell 1989; Whyte et al. 1998). The products of these pathways are primary alcohols, monocarboxylic fatty acids, dicarboxylic fatty acids, secondary alcohols and ketones.

This paper presents the characterization of intra- and extracellular fatty acids produced by a *Rhodococcus* sp., isolated from an oil-contaminated tropical environment. When grown on even carbon alkanes the organism releases only dicarboxylic fatty acids into the medium and on odd carbon alkanes it releases only monocarboxylic fatty acids.

Materials and methods

Materials

Assam crude oil was obtained from Oil and Natural Gas Commission (ONGC), India. All reagents and chemicals were of analytical grade.

Methods

Enrichment and isolation of crude oil degrading organism

Oil-contaminated samples from coastal regions near Mumbai were enriched on seawater supplemented with 38 mM ammonium sulfate and 0.1 mM dipotassium hydrogen orthophosphate at pH 8.0 with 1% (w/v) Assam crude oil. Flasks were incubated at 30 °C at 150 rpm for 5 days. After two transfers into the same medium, fresh aliquots of the organisms were streaked onto the seawater nutrient agar plates containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 2.5% (w/v) agar, pH 8.0. The plates were incubated at 30 °C. Colonies isolated from these plates were tested for their ability to degrade crude oil using 1% (w/v) Assam crude oil. Initial testing was done by gravimetry after extraction of residual crude oil in chloroform, the per cent degradation of crude oil being determined by reference to an uninoculated control extracted under similar conditions. The best degrader was selected for further studies. All subsequent analyses of residual crude oil or supplied alkane hydrocarbons were done on a gas chromatograph.

Analysis of the aliphatic fraction of crude oil

The aliphatic fraction of residual crude oil, separated by the method of Atlas (1975), was suspended in n-hexane and concentrated to 2 ml. One μl was injected into a Shimadzu GC-RIA gas chromatograph fitted with SE-30 column, coated with Chromosorb W. The carrier gas was nitrogen. Detection was by flame ionization. Temperature was held at 60 °C for one min and programmed to 250 °C at a rate of 6 °C per min where it was held for 15 min. Injector temperature was 250 °C. Quantitation was done in accordance with Dibble and Bartha (1976).

Degradation of various hydrocarbons by the isolate

Degradation of specific n-alkanes, kerosene and pristane (2,6,10,14- tetramethylpentadecane) were estimated by extracting residual hydrocarbon from the test and control flasks with 200 ml chloroform and concentrating the extracts to 50 ml. One μl was injected into a Shimadzu GC-RIA gas chromatograph with isothermal programming at 150 °C. The difference in area under the peaks between control and tests were reported as per cent degradation of that compound.

Identification of the isolate

The organism was grown on Tryptose soybean agar (1.5% (w/v) tryptone, 0.5% (w/v) soya peptone, 85 mM sodium chloride, 1.5% (w/v) agar, pH 7.5) at 30 °C for 24 h. Fatty acids were extracted by saponification (sodium hydroxide and methanol) and methylated with acidic methanol followed by solvent extraction (hexane and methyl tertiary butyl ether), a protocol given by the manufacturers of the Microbial Identification System (MIS, Microbial Identification Inc.®, USA). The fatty acid methyl esters (FAME) were analyzed on the MIS system with hydrogen as a carrier gas and FID detector, programmed at 5 °C per min from 170–270 °C and a 2 min constant temperature at 270 °C. The isolate was tentatively identified as an actinomycete. Genus level identification of the organism was carried out by testing for the presence of diaminopimelic acid (DAP) (Becker et al. 1964), characteristic sugars (Becker et al. 1965) and mycolic acids (Minnikin et al. 1975). Biochemical tests were carried out in accordance with Goodfellow & Alderson (1977) and Goodfellow (1989). Assimilation of sugars was checked using minimal agar medium, prepared in distilled water, supplemented with 35 mM urea, 0.1 mM dipotassium hydrogen orthophosphate, 85 mM sodium chloride and 1% (w/v) test sugar as sole source of carbon.

DNA was extracted by the method of Mordarski et al. (1976) and the T_m was determined using the method of Marmur & Doty (1962). Mol% G + C content was calculated using the formula of Mandel & Marmur (1968), G + C = 2.44 ($T_m - 53.9$).

Morphological features of the isolate were studied using a Leica Stereoscan 440 model of Scanning Electron Microscope (M/s Leica Cambridge Ltd. UK). 18 h old slide cultures were fixed in glutaraldehyde (2% v/v) for 18 h and serially dehydrated by ethanol (10–95% v/v). The samples were mounted on specimen-mounting stubs by using conducting silver paste and then coated with a thin layer of gold in Polaron coating unit E5000 to prevent charging of the specimens.

The organism was grown on Glucose yeast extract agar (28 mM glucose; 0.5% (w/v) meat extract; 0.5% (w/v) peptone; 0.5% (w/v) yeast extract; 2% (w/v) agar) and Sauton's agar (30 mM L-asparagine; 20 mM magnesium sulfate, 7H2O; 9.5 mM citric acid; 2.5 mM dipotassium hydrogen orthophosphate; 0.005% (w/v) ferric ammonium citrate; 4 ml glycerol; 2% (w/v) agar) and morphology and colony characteristics of the organism were checked.

Cellular fatty acids of the organism grown on various carbon sources

Fatty acids of fructose-grown cells and odd and even carbon number alkane-grown cells were extracted as described by Makula & Finnerty (1968). For this purpose, about 100 ml culture broth was centrifuged (10,000 rpm, 10 min) and in each case, 75 mg wet weight of the cells was used. Fatty acid methyl esters were prepared by refluxing the cell extracts with methanol and concentrated sulfuric acid for 4 h at 100 °C (Zuckerberg et al. 1979). The final volume of the esters in chloroform was 100 μl. The esters were analyzed on a Shimadzu GC-MS QP5000 on a 30 m SE-30 capillary column. Injector and detector were at 250 °C. Initial temperature of the column was maintained at 140 °C for 2 min followed by a temperature increase of 20 °C per min. Final temperature was 250 °C, held for 10 min. The carrier gas was helium, injector mode split ratio was 70. The fragments were identified by comparison with data stored in NIST62.lib, software available with Shimadzu GC-MS QP5000. The relative percentage of each fatty acid was determined by comparing the area of each individual fatty acid peak to the total area.

Extracellular products of the organism grown on hydrocarbons

Extracellular products of growth on n-tridecane, n-tetradecane, n-hexadecane, n-heptadecane and pristane were determined on cell-free broth, made emulsifier-free by ultrafiltration through Amicon YM-10 membrane, under nitrogen, at 15 °C. The pH of the filtrate was adjusted 10.0 with 1N sodium hydroxide and extracted with 3 volumes of hexane to remove residual alkane. The aqueous phase containing sodium salt of fatty acids was acidified to pH 2.0 and then extracted with chloroform. The chloroform extract was concentrated by rota evaporation and was refluxed with methanol and concentrated sulfuric acid to give methyl esters as described earlier. Fatty acid methyl esters were identified on a Shimadzu GC-MS QP5000, as given above.

Results and discussion

The isolate was a Gram positive, non-motile, oxidase negative, catalase positive rod, which produced orange coloured, convex colonies with entire margins. Qualitative analysis of cellular fatty acids on a Microbial Identification System (Hewlett Packard Gas Chromatograph 5890), showed the presence of saturated and unsaturated fatty acids and tuberculostearic acid suggesting that the isolate was a nocardioform actinomycete (Goodfellow & Lechevalier 1989; Goodfellow 1989). Whole cell hydrolysate showed only meso-DAP placing it in the *Nocardia* group (Becker et al. 1964). Arabinose and galactose were the characteristic sugars (Becker et al. 1965). Presence of meso-DAP, arabinose, galactose and mycolic acid suggested that this organism was a type IV actinomycete. The calculated mol% G + C was 69, which was within the range for the genus *Rhodococcus* (Finnerty 1992). Furthermore, the isolate contained C_{16}, $C_{16:1}$, C_{18}, $C_{18:1}$ fatty acids regardless of the growth substrate. As shown by Goodfellow (1989), the *Rhodococcus* group constitutively contains these fatty acids.

Only rod forms were seen at lag, exponential and stationary growth phases in cultures of this organism suggesting that it was an amycelial strain of *Rhodococcus* (Figure 1). In its ability to utilize specific carbon sources it closely resembled *Rhodococcus terrae* and *Rhodococcus bronchialis* (Goodfellow, 1989) showing 17 matches out of 23 with both these species (data not shown). On glucose yeast extract agar and Sauton's medium, *R. bronchialis* forms rough brownish colonies and synnemata of vertically arranged filaments on the surface of colonies after 12–18 h incubation (Goodfellow 1989). *R. terrae* on the other hand produces pink to orange colonies on both media, which the present isolate also does. It appears that the actinomycete may be *R. terrae* although it was isolated from a marine environment. It has been deposited as *Rhodococcus* sp. NCIM 5126 in the National Collection of Industrial Microorganisms (NCIM) at the National Chemical Laboratory (NCL).

The organism degraded up to 50% of the aliphatic fraction of Assam crude oil in seawater supplemented with 35 mM nitrogen as urea and 0.1 mM phosphorus as dipotassium hydrogen orthophosphate, after 72 h at 30 °C and 150 rpm. Maximal degradation of 94% of the supplied n-tetradecane was obtained (Table 1). High carbon number alkanes were degraded to less extent and only 5% of eicosane was degraded in 72 h. The organism degraded 92% of the supplied kerosene, which contained primarily n-C10 to n-C13 alkanes. It also degraded pristane (2,6,10,14-tetramethylpentadecane) (Table 1). That *Rhodococcus* strains degrade hydrocarbons between n-C12 to n-C_{20} is well known (Sorkhoh et al. 1990). Whyte et al. (1998) and Milekhina et al. (1998) have also re-

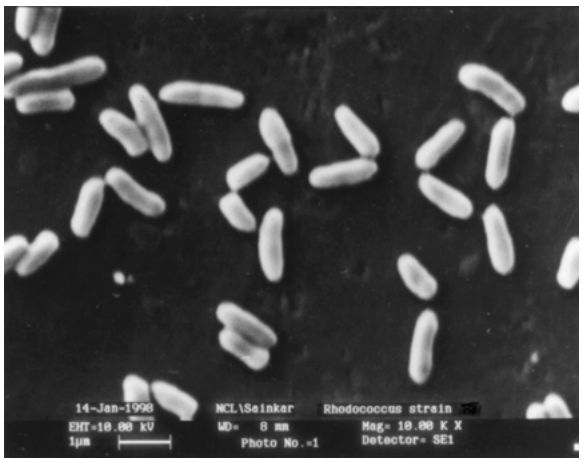

Figure 1. Scanning Electron Micrograph (SEM) of *Rhodococcus* sp. NCIM 5126 showing rods.

Table 1. Degradation of various hydrocarbons by *Rhodococcus* sp. NCIM 5126, analyzed by Gas chromatography, as described in Materials and Methods

Substrate	Amount supplied (%)	Amount supplied (mg)	Degradation (%)	Degradation (mg)
n-Tridecane	1	500	80	400
n-Tetradecane	1	500	94	470
n-Hexadecane	1	500	75	375
n-Heptadecane	1	500	50	250
n-Ecisonae	1	500	5	25
Kerosene	1	500	92	460
Pristane	1	500	30	150

ported degradation up to n-C30 by various strains of *Rhodococcus*. Nakajima et al. (1985) reported pristane degradation by *Rhodococcus* sp. BPM 1613.

The relative percentage of intracellular fatty acids was higher in hydrocarbon grown cells compared to fructose grown cells (Table 2). It is probable that the composition of cellular membranes change in response to the supplied carbon source. The increased lipophilicity may facilitate uptake and intracellular transport of hydrocarbons. Whyte et al. (1999) have earlier reported that compared to glucose-acetate grown cells, a psychrotrophic *Rhodococcus* sp. was more lipophilic when grown on n-hexadecane or diesel fuel. In the present organism, besides the constitutively present fatty acids, other cellular fatty acids corresponded to the supplied hydrocarbon source (Table 2). It has been suggested that

Table 2. Relative percentage of the cellular fatty acids of *Rhodococcus* sp. NCIM 5126, grown on various carbon sources, analyzed on GC–MS, identified by comparison with the data stored in NIST62.lib library

Fatty acid	Growth substrate					
	Fructose	n-C_{13}	n-C_{14}	n-C_{16}	n-C_{17}	Pristane
C13	–	25.36	–	–	–	–
Br^1–°C_{13}	–	–	–	–	–	6.02
C_{14}	–	–	30.26	–	–	–
C_{15}	–	–	–	–	–	10.02
Br–C_{15}	–	–	–	–	–	24.24
C_{16}	12.90	12.52	6.48	33.23	5.27	4.13
$C_{16:1}$	5.04	5.78	5.30	6.23	5.20	5.05
C_{17}	–	–	–	–	30.59	3.04
Br–C_{17}	–	–	–	–	–	3.03
C_{18}	6.69	6.53	5.03	5.78	5.14	0.20
Br–C_{18}	–	–	–	–	–	0.25
$C_{18:1}$	6.97	6.67	6.95	6.76	6.16	0.36
Total	31.60	56.86	54.02	52.00	52.36	56.34

Br^1 – branched.

'intact/direct incorporation', of fatty acids into cellular lipids occurs in alkane degrading strains (King & Perry 1975). When grown on even carbon number alkanes the cells contained only even carbon number fatty acids, for example, growth on n-tetradecane resulted in the production of a substantial quantity of intracellular tetradecanoic acid. On odd carbon number alkanes the corresponding monocarboxylic fatty acids were predominant. Hug & Fiechter (1973) and Yanagawa et al. (1972) have reported similar results with *Candida tropicalis* and *Corynebacterium simplex* respectively. Pristane-grown cells contained 2,6,10,14-tetramethylpentadecanoic acid as a major cellular fatty acid.

Only monocarboxylic acids were produced extracellularly by this organism when grown on odd carbon number alkanes and only dicarboxylic acids were produced when grown on even carbon number alkanes (Table 3). These data suggest the operation of a diterminal oxidation pathway, in addition to monoterminal oxidation of n-alkanes. The mechanism by which the organism switches production is as yet unknown. Earlier, Woods & Murrell (1989) showed that *Rhodococcus rhodochrous* PNKb1 utilized propane by both the terminal and subterminal pathways producing propan-1-ol, propan-2-ol as important intermediates. *Rhodococcus erythropolis* ATCC 4277 degraded n-C5 to n-C_{16} alkanes only by the subterminal pathway (Ludwig et al. 1995) whereas

Table 3. Extracellular fatty acids of alkane metabolism by *Rhodococcus* sp. NCIM 5126, analyzed on GC-MS, identified by comparison with the data stored in NIST62.lib library

Alkane growth substrate	Extracellular fatty acids
n-Tridecane	Undecanoic acid, Hexadecanoic acid
n-Tetradecane	Undecanedioic acid
n-Hexadecane	Undecanedioic acid, Tridecanedioic acid
n-Heptadecane	Hexadecanoic acid
Pristane	2,6,10,14-Tetramethylpentadecanoic acid

Corynebacterium strain 7E1C produced only dodecanedioic acid from n-dodecane and tetradecanedioic acid from n-tetradecane (Broadway et al. 1993). Recently, Whyte et al (1998) reported both subterminal oxidation and monoterminal oxidation of n-C12 and n-C_{16} by *Rhodococcus* sp. strain Q15 producing 1-dodecanol and 2-dodecanone and 1-hexadecanol and 2-hexadecanol respectively.

Pristane oxidation has been reported in the *Rhodococcus* group of organisms. Production of both monoic acids and dioic acids of pristane by *Rhodococcus* sp. BPM 1613 has been reported by Nakajima et al. (1985). Prinik et al. (1974) reported similar results for *Brevibacterium erythrogens*. In the present case pristane grown cells produced only 2,6,10,14-tetramethylpentadecanoic acid suggesting the occurrence of only monoterminal oxidation pathway for pristane conversion by this organism.

Conclusions

Rhodococcus sp. NCIM 5126, isolated from the chronically oil-polluted coastal region near Mumbai, was able to degrade 50% of the aliphatic fraction of Assam crude oil, alkanes up to n-C_{20}, mixed chain alkane kerosene and the branched chain alkane pristane. When grown on various alkanes, the major cellular fatty acids corresponded to the chain length of the supplied hydrocarbon. The relative percentage of cellular fatty acids was more for alkane-grown cells compared to fructose grown cells and this increased lipophilicity may facilitate uptake and intracellular transport of hydrocarbons. The degradation products of hydrocarbon metabolism by this organism suggested monoterminal oxidation of hydrocarbons as the major pathway, however the presence of dioic acids also suggested the occurrence of a diterminal oxidation pathway.

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