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SHORT COMMUNICATION

Degradation of oil tank sludge using long-chain alkane-degrading bacteria

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Abstract Bacteria degrading a very long-chain alkane, *n*-tetracosane, were isolated from enrichment culture of soil in Okinawa. Phylogenetic analysis of their16S rRNA sequences revealed that they belong to classes Gammaproteobacteria and Actinomycetes. Three isolates belonging to the genera *Acinetobacter* sp., *Pseudomonas* sp., and *Gordonia* sp. showed a stable growth on *n*-tetracosane and had a wide range of assimilation of aliphatic hydrocarbons from C_{12} to C_{30}, while not on alkanes shorter than C_8. Of the isolates, *Gordonia* sp. degraded oil tank sludge hydrocarbons efficiently by solving the sludge in a hydrophobic solvent, while *Acinetobacter* sp. showed little degradation, possibly due to the difference in the mechanism of hydrophobic substrate incorporation between proteobacteria and actinobacteria. The data suggested that non-heme di-iron monooxygenases of the AlkB-type, not bacterial CYP153 type cytochrome P450 alkane hydroxylase, was involved in the alkane degradation.

Keywords *n*-alkane · Oil tank bottom sludge · Biodegradation · Actinobacteria · *Gordonia* sp.

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Petroleum is a complex mixture of non-aqueous and hydrophobic components like *n*-alkane, aromatics, resins. and asphaltenes. Bioavailability might be the limiting factor controlling the biodegradation of such compounds. Among the petroleum fractions, oil tank bottom sludge (OTBS) has been regarded as a hazardous and recalcitrant petroleum-derived substance. In addition, the oily wastes in crude storage tank bottoms are periodically removed, posing difficulties for disposal (Ferrari et al. 1996). In the past, these wastes were disposed of after mixing them with soil and partially stabilizing them with additives such as magnesite; in contrast, nowadays, they are generally detoxified using expensive physical–chemical processes. Therefore, biotreatments should be considered as an alternative, not only to decrease the volume of waste but also to reduce their toxicity. Bioreactors, bioslurry techniques, landfarming treatments, or composting comprise bioremediation alternatives for OTBS and for hydrocarbon wastes in general (Marin et al. 2006). Microbial degradation of aliphatic hydrocarbons have been investigated with the aim of bioremediation for petroleum-polluted areas. For example, *n*-alkane in the petroleum sludge degradation by a bacterial consortium was reported to be enhanced by nutrients and biosurfactant addition (Rahman et al. 2003). Pure cultures, including proteobacteria (*Acinetobacter* sp., *Pseudomonas* sp., and *Stenotrophomonas* sp.) and firmicutes (*Bacillus* sp.), have also been reported to degrade petroleum oily sludge (Cerqueira et al. 2011; Verma et al. 2006), while little information is available on sludge degradation with actinobacteria, such as *Gordonia* sp. and *Rhodococcus* sp., well known as hydrocarbon degraders (Arenskoetter et al. 2004), especially for the degradation of alkanes with carbon number higher than 22. Gallego et al. (2007) examined oil tank bottom sludge degradation using microbial consortia, showing that a consortium containing *Acinetobacter* spp., *Nocardioides* sp., *Stenotrophomonas* sp., *Pseudomonas* sp., *Aeromonas* sp., and *Rhodotorula* spp. resulted in a good cometabolic effects,

emulsification properties, colonization of oil components, and degradative capabilities. In the scientific literature on microbial alkane degradation, *n*-alkanes (linear alkanes) have frequently been referred to as short-chain (C_1 to C_4), medium-chain (C_5 to C_9), long-chain (C_{10} to C_{17}), and very long-chain *n*-alkanes (more than C_{18}), according to the length of their linear carbon chain. In addition to the extensive research in microbial degradation of *n*-alkanes with carbon number less than 18, several reports on very long-chain *n*-alkanes degradation can be found (Wentzel et al. 2007). For many *n*-alkane-degrading bacteria, multiple alkane hydroxylases have been reported exhibiting overlapping substrate ranges, including integral membrane non-heme di-iron monooxygenases of the AlkB-type (Smits et al. 1999) and cytochrome P450 alkane hydroxylases of the CYP153 family (van Beilen et al. 2006).

Here, we describe the isolation of bacteria capable of degrading recalcitrant, long-chain aliphatic hydrocarbons, such as *n*-tetracosane, followed by their application for laboratory scale OTBS remediation.

Bacterial strains used in this study were isolated by enrichment culture containing *n*-tetracosane (CH_3-$(CH_2)_{22}$-CH_3) as the sole carbon and energy source from 500 soils from Okinawa main islands. For the enrichment and alkanes degradation test, basal mineral salts medium No. 11 was used, as described previously (Matsui et al. 2009). Enrichment culture was perfomed aerobically at 30 °C, 150 spm with reciprocal shaker (Taitec, Tokyo, Japan), for 2 weeks, followed by the repeat of transferring 5 % into the fresh medium for 3 times. The obtained enrichment culture was further spread on nutrient agar (Oxoid, Hampshire, UK) supplemented with glucose for colony isolation.

General DNA manipulations were performed as described by Sambrook and Russell (2001). Chromosomal DNA from the isolated strains were extracted as described previously (Matsui et al. 2009) and used for polymerase chain reaction (PCR). The 16S rRNA gene, alkB, and cytochrome P450 hydroxylase of the CYP153 family homologue gene were amplified by PCR using EX Taq DNA polymerase (Takara Bio, Shiga, Japan) and a thermal cycler (type PC-818; Astec, Fukuoka, Japan), as described previously (Matsui et al. 2009; Smits et al. 1999; van Beilen et al. 2006). The PCR-amplified 16S rRNA gene (approximately 1.5 kb long), alkB gene (0.5 kb), and cytochrome P450 hydroxylase gene (0.3 kb) fragments were purified by agarose gel electrophoresis, ligated into the pT7 blue vector (Novagen Merck Milllipore, Darmstadt, Germany), and used for the transformation of *Escherichia coli* JM109 (Takara Bio). The nucleotide sequences of both strands of the cloned genes were determined by using the M13-47 and RV-M primers (Novagen Merck Millipore) and the ABI model 3100 and BigDye terminator kit, v.1.1 (Applied Biosystems, CA, USA) according to the manufacturer's instructions.

Growth of bacterial cells was estimated by measuring numbers of viable colonies after serial dilution of culture broths with sterilized saline solution, expressed as colony forming units (CFU) per unit of volume. Substrate consumption was analyzed by gas chromatography (GC) after extracting the reaction solution with *n*-hexane using n-tetradecane as the internal standard. The gas chromatograph, (Shimadzu GC-14A; Shimadzu, Kyoto, Japan) was fitted with a DB-5 capillary column (length 30 m; Agilent technologies, CA, USA). Run conditions were as follows: start temperature 100 °C 10 °C /min ramp rate to a final temperature at 250 °C for 5 min. For OTBS analysis, the extraction was with an equal volume of *n*-hexane, followed by the GC analysis at starting temperature of 80 °C for 4 min, 5 °C/min ramp rate to a final temperature at 300 °C for 52 min. Biosurfactant production by the isolates were evaluated as the emulsification activity of their culture broth as described by Saeki et al. (2009). In order to measure the emulsification activity of the biosurfactant, 50 μl soybean oil and 1 ml

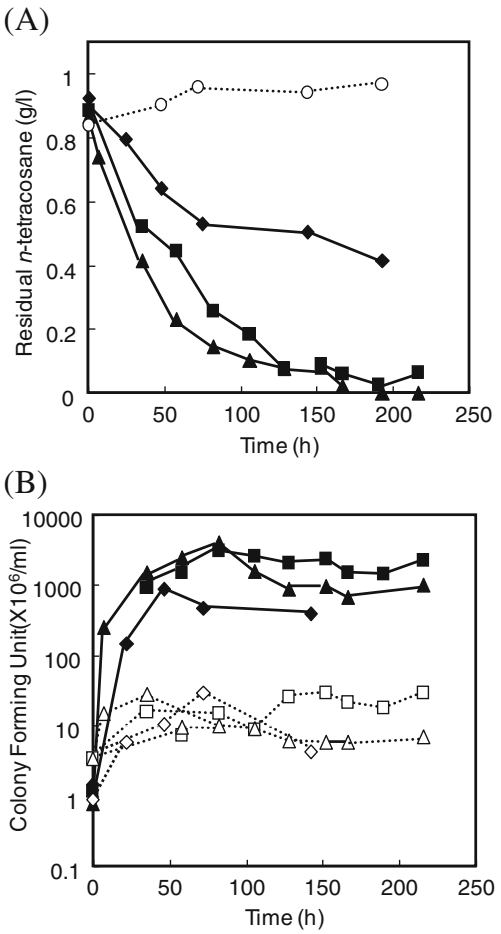

Fig. 1 Time course of *n*-tetracosane degradation by the isolates. **a** Residual *n*-tetracosane (g/l), **b** cellular growth (CFU/ml). *Triangles Acinetobacter* sp. 49A, *squares Pseudomonas* sp. 11A, *diamonds Gordonia* sp. 30A. *Dotted lines* results in the absence of cells (**a**), and in the absence of substrate (**b**)

Table 1 Characterization of n-tetracosane (n-C_{24}) degradation by the isolates

	n-C_{24} degradation (%)[a]				
	No solvent	HMN[b]	Pristane	Squalane	BS (g/l)[c]
Pseudomonas sp. strain 11A	71.5	44.1	0	87.5	0.18
Gordonia sp. strain 30A	88.3	100	67.3	100	n.d.[d]
Acinetobacter sp. strain 49A	91.2	37.4	36.5	0	0.19

[a] n-C24 degradation in the presence of 10 % (v/v) solvent

[b] 2,2,4,4,6,8,8-Heptamethylnonane

[c] Biosurfactant production when grown with n-C24 as the sole carbon source

[d] Not detected

biosurfactant sample were added to 9 ml of 50 mM phosphate buffer (pH 7.0) taken in an φ18-mm×125-mm test tube. The test tube was vigorously shaken at 345 rpm for 10 min at 30 °C. Thereafter, the optical density was measured at 620 nm using the UV–photometer mini photo 518R (Taitec). The emulsion turbidities of different concentrations (0–20 mg/l) of the biosurfactant sample were directly proportional to the concentrations of the sample. To estimate the total concentration of the biosurfactant in the culture supernatant, the emulsification activity of the culture supernatant was measured and then calculated with respect to soybean oil on the basis of the standard curve that was obtained by using the data of the standard biosurfactant. The composition of OTBS, analyzed by the TLC-FID method, modified as described by Cebolla et al. (1995), revealed that it consisted of 37.7±1.1 % of saturates, 40.2±2.1 % of aromatics, 18.5±0.8 % of resins, and 3.5±0.3 % of asphaltenes.

OTBS was kindly provided by Akita Oil Storage, Akita, Japan Various alkanes were supplied by Tokyo Kasei Kogyo, Tokyo, Japan. All other materials were of the highest purity commercially available and were used without further purification.

Sequence of fragments of 16S rRNA and alkB gene of the isolates have been assigned DDBJ/EMBL/GenBank accession nos. AB638843–AB638848 and AB638866–AB638868, respectively.

By the enrichment culture with 3 times transfer on *n*-tetracosane as the sole carbon and energy source, 40 soil samples out of 500 exhibited a stable bacterial growth. Eighteen bacterial strains were selected among the isolates from the enrichment culture broths, followed by assimilating tests of the substrate in liquid cultures. Analysis of the 16S rRNA sequences for the isolates revealed that 10 belonged to *Acinetobacter* sp., 3 to *Pseudomonas* sp., and 5 to *Gordonia* sp. Of the isolates, 3 strains (namely 11A,

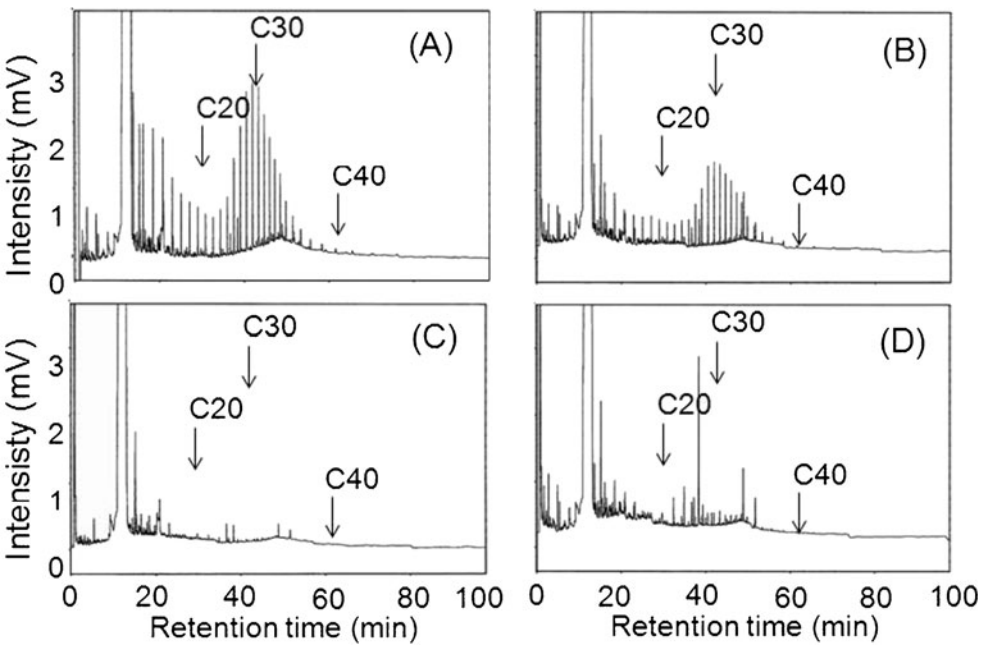

Fig. 2 Gas chromatograms of the HMN layer after cultivation with OTBS for 7 days in the presence of pristane. **a** No cells inoculated, **b** *Pseudomonas* sp. 11A, **c** *Gordonia* sp. 30A, and **d** *Acinetobacter* sp. 49A. Peaks at around 10 min were derived from the added HMN and its impurities. *Arrows* indicates peaks for linear alkanes carbon number

30A, and 49A, belonging to *Pseudomonas* sp., *Gordonia* sp., and *Acinetobacter* sp., respectively) were selected from the each genera, as showing stable growth on *n*-tetracosane as the carbon source, and subjected for further studies.

Figure 1 shows the typical time courses of *n*-tetracosane degradation by the selected isolates. *Acinetobacter* sp. and *Pseudomonas* sp. degraded more than 90 % of *n*-tetracosane in 120 h of incubation. *Gordonia* sp. degraded nearly 50 % at 60 h, while showing comparable cellular growth to that of the two other strains. They assimilated *n*-alkanes ranging from C_{14} to C_{30}, although *n*-alkanes shorter than C_8 did not support the growth of all the strains (data not shown).

In many bacterial species, the oxidation of alkanes is catalyzed by non-heme di-iron monooxygenase Alk systems (van Beilen and Funhoff 2005), although different pathways have been identified (van Beilen et al. 2006). From the genome of all the 3 strains, using degenerated primers, PCR products of the expected size were obtained for the alkB gene. No amplification was observed for the cytochrome P450, suggesting that only non-heme di-iron alkane hydroxylase could be involved in the degradation. Sequence analysis of the alkB gene homologue obtained from the 3 strains' genome revealed each one belongs to the same families as those classified from 16S rRNA sequences. Homology in the primary sequences from 11A, 30A, and 49A strains with the closest proteins were 99.4 (*Pseudomonas aeruginosa* PAO1, reference No. 2618382EFW), 100 (*Gordonia amarae* DSM43392, D1MBU3) and 97.3 % (*Acinetobacter calcoaceticus* PHEA-2, F0KMZ3), respectively. Roles of these genes in hydrocarbons degradation should further be examined.

To facilitate the fluidity of OTBS to enhance its degradation, since no degradation was observed when cultured with the oil tank sludge as the sole source of carbon (data not shown), water/solvent two-phase cultivation was further examined with the 3 strains studied (Table 1). To examine the effect of solvents for enhancement of the substrate solubility, we used heptamethyl-nonane (HMN), pristane, and squalane as solvents for the enhancement of solubility of OTBS, because they are known as biocompatible and resistant to microbial attack (Hori et al. 2002; Muñoz et al. 2008). As shown in Table 1, *P. aeruginosa* and *Acinetobacter* sp. showed no degradation in the presence of pristane and squalane, respectively, while significant degradations were observed in the presence of all the solvents tested when using *Gordonia* sp., resulting in *n*-tetracosane degradation of 100, 67.3, and 100 % when using HMN, pristane, and squalane, respectively. Production of surface-active compounds (bioemulsifiers or biosurfactant) was detected only in *Pseudomonas* sp. strain 11A and *Acinetobacter* sp. 49A, but not in *Gordonia* sp. 30A (Table 1). The effect of HMN addition on hydrocarbon degradation was further examined for OTBS degradation as shown in Fig. 2. Significant alkanes degradation was observed only in the case of growing cultures of *Gordonia* sp. in the presence of HMN, while no degradation was obtained with *Pseudomonas* sp., and less degradation with *Acinetobacter* sp. Microbial incorporation/degradation of petroleum-derived hydrocarbons were started by two possible mechanisms, emulsification of the oily droplets or growth in the droplets due to the hydrophobic cell surface (Monticello 2000). Based on these facts, efficient degradation of OTBS by *Gordonia* sp. in the presence of HMN could be explained by the latter mechanism, since the strain degraded efficiently in the presence of the solvent although no biosurfactant production was observed.

In this study, efficient OTBS degradation was shown using a new isolated *Gordonia* sp., in the presence of a hydrophobic solvent. This character might have a potential for efficient sludge degradation by combination with a conventional physico-chemical tank sludge treatment process such as crude oil washing (Mitchel 1994), which is widely used for OTBS removal from the tank.

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