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NOTE TO THE EDITOR

Isolation of alkane-degrading bacteria from deep-sea Mediterranean sediments

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Abstract

Aims: To isolate and identify alkane-degrading bacteria from deep-sea superficial sediments sampled at a north-western Mediterranean station.
Methods and Results: Sediments from the water/sediment interface at a 2400 m depth were sampled with a multicorer at the ANTARES site off the French Mediterranean coast and were promptly enriched with Maya crude oil as the sole source of carbon and energy. Alkane-degrading bacteria belonging to the genera *Alcanivorax*, *Pseudomonas*, *Marinobacter*, *Rhodococcus* and *Clavibacter*-like were isolated, indicating that the same groups were potentially involved in hydrocarbon biodegradation in deep sea as in coastal waters.
Conclusions: These results confirm that members of *Alcanivorax* are important obligate alkane degraders in deep-sea environments and coexist with other degrading bacteria inhabiting the deep-subsurface sediment of the Mediterranean.
Significance and Impact of the Study: The results suggest that the isolates obtained have potential applications in bioremediation strategies in deep-sea environments and highlight the need to identify specific piezophilic hydrocarbon-degrading bacteria (HCB) from these environments.

Deep-sea areas are the site of a growing proportion of world oil production. However, little information is available on the active role played by hydrocarbon-degrading bacteria (HCB) in these environments. While studies on HCB inhabiting coastal areas are increasing, little is known about their deep-sea subsurface counterparts (Cui *et al.* 2008). To date, few HCB have been isolated from deep-sea sediments (Schwarz *et al.* 1974; Liu and Shao 2005; Xu *et al.* 2008). The aim of the study was to isolate and identify HCB from deep-sea superficial sediments sampled at a north-western Mediterranean station.

The sediment from the water/sediment interface at 2400 m was collected with a multicorer sampler on 11 May 2007 at the ANTARES site (42°50′N 6°10′E), 20 nautical miles (37 km) from Toulon. The core was tightly enclosed in the core tube after insertion, and protected from disturbance from the overlying sea water by an automatic mechanism closing the ends. A first blank coring was carried out to wash the core tubes with *in situ* sediments.

The synthetic sea water (SSW) medium used for enrichment contained (per litre of distilled water) 2 g Tris-(hydroxy-aminomethane), 23 g NaCl, 0·75 g KCl, 1 g NH_4Cl, 6·16 g $MgSO_4.7H_2O$, 5·08 $MgCl_2.6H_2O$ g and 1·5 g $CaCl_2$ (pH 7·7), autoclaved at 120°C for 20 min. Sterile solutions of $FeSO_4$ and PO_4 were added before incubation (final concentration 0·2 and 2 mmol l^{-1}, respectively). Solid Complex Medium (SCM) was made from (per litre of SSW) 20 g microbiology agar-agar (bioMérieux, Marcy l'Etoile, France), 5 g pastone (AES Laboratoire, Combourg, France) and 200 mg yeast extract (Difco).

Sediments from the first 5 cm of the central part of the cores were sampled using an end-cut sterile 50-ml syringe and kept at 4°C in sterile Falcon tubes until use. Sediment sample (20 g) was mixed with 50 ml of SSW and sterile 1 g l^{-1} *Maya* crude oil in a 250-ml Erlenmeyer

flask and was agitated in the dark on a reciprocal shaker (96 rev min^{-1}) at 20 ± 1°C for 10 days. Two-millilitre aliquots were transferred six times from the mixed culture to fresh medium under the same conditions. Serial dilutions of mixed culture (0·1 ml) were streaked onto agar plates prepared with SCM. Sterile filter soaked with *n*-pentadecane, *n*-hexadecane and *n*-heptadecane (Sigma, Lisle d'Abeau Chesnes, France) were attached on the upper side of these plates. The plates were incubated at 20 ± 1°C in the dark for 30 days. Single colonies were picked out and purified by multiple replicates from plate to plate. From the various clones isolated, 13 grew on alkanes (hereafter referred as strains ANT-2400 S1 to ANT-2400 S13) and were used for biodegradation assays.

Isolates were inoculated individually in triplicate SSW supplemented with sterile hexadecane, heptadecane and eicosane (1 g l^{-1} each). After 30 days of incubation, residual hydrocarbons were extracted by liquid–liquid extraction using dichloromethane (Rathburn Chemicals Ltd, Walkerburn, UK) for 16 h and were quantified by external calibration with authentic standards using a Perkin Elmer AutoSystem XL Gas Chromatograph equipped with a flame ionization detector (GC-FID) and a HP-5 capillary column (25 m × 0·32 mm i.d. × 0·52 μm film thickness; J & W Scientific, Folsom, CA). The oven temperature was programmed to increase from 70 to 150°C at 15°C min^{-1}, 6°C min^{-1} to 320°C, at which it was held for 10 min.

Isolated strains genomic DNA was extracted using a Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The 16S rRNA genes were amplified using the universal primer set 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′). The thermal cycling parameters were 5 min at 94°C, 35 cycles of denaturation for 1 min at 94°C, 1 min at 50°C, 2 min at 72°C and 10 min at 72°C. Amplification reactions were performed using an automated DNA thermocycler iCycler (Bio-Rad Laboratories, Hercules, CA). Purified products were cloned into pGEM-T Easy Vector System (Promega, Charbonniéres-Les-Bains, France) and transformed into *Escherichia coli* JM109 high-efficiency competent cells following the manufacturer's instructions. Plasmids were isolated (Promega Wizard™ Plus Minipreps) and sequenced (GATC Biotech, Konstanz, Germany, http://www.gatc-biotech.com/en/index.html). The amplified DNA fragments were analysed online with BLAST in the GenBank databases where sequences were submitted. Isolated strains were assigned accession numbers GQ153637 to GQ153649.

Identification based on 16S rRNA sequences from the 13 HCB isolates showed an interesting similarity to the results of Cui *et al.* (2008) in that the majority of isolates belonged to *Alcanivorax* (Table 1). Gram-positive genera and *Alcanivorax venustensis*-affiliated strains preferred to degrade *n*-hexadecane (40–63%), while for the other genera, *n*-heptadecane appeared to be the most degraded alkane (8–44%). *Alcanivorax dieselolei* and *Marinobacter* sp. did not degrade *n*-hexadecane under our test condition and analytical procedure used (GC-FID). This was confirmed by incubating these isolates using *n*-hexadecane (1 g l^{-1}) as the sole source of carbon and energy. The preference of one substrate over another has previously been observed in HCB even in *Alcanivorax*-affiliated

Table 1 Percentage of model hydrocarbons degraded by the different alkane-degrading bacterial isolates after 30 days of incubation at 20°C. Values shown are mean of triplicates followed by standard deviation. *n*-C_{16} = hexadecane, *n*-C_{17} = heptadecane, *n*-C_{20} = eicosane

Isolates (accession no.)	Closest relative in GenBank database (% identity range)	% degradation (1 g l^{-1} of substrate) ± SD		
		n-C_{16}	*n*-C_{17}	*n*-C_{20}
Gram-negative (gamma subclass of *Proteobacteria*)				
ANT-2400 S3 (GQ153639)	*Alcanivorax dieselolei* PR56-2 EU440990 (98%)	0%	26·3% ± 2·2	4·2%
ANT-2400 S5 (GQ153641)	*A. dieselolei* PR56-2 EU440990 (99%)	0%	15·8% ± 0·9	8·5% ± 0·6
ANT-2400 S6 (GQ153642)	*A. dieselolei* PR56-2 EU440990 (99%)	0%	25·2% ± 4·5	0%
ANT-2400 S7 (GQ153643)	*A. dieselolei* PR56-2 EU440990 (98%)	0%	8·2% ± 1·8	7% ± 1
ANT-2400 S8 (GQ153644)	*A. dieselolei* PR56-2 EU440990 (96%)	0%	26·7% ± 1·4	18·8% ± 1·4
ANT-2400 S11 (GQ153647)	*A. dieselolei* PR56-2 EU440990 (98%)	0%	35·2% ± 0·7	5·1% ± 0·5
ANT-2400 S12 (GQ153648)	*A. dieselolei* PR56-2 EU440990 (99%)	0%	40·1% ± 0·9	15·8% ± 0·3
ANT-2400 S4 (GQ153640)	*Alcanivorax venustensis* PR51-8 EU440952 (96%)	48·7% ± 7·6	44·6% ± 5·2	34·4% ± 5·0
ANT-2400 S1 (GQ153637)	*Marinobacter* sp. H96B3 FJ746575 (99%)	0%	15·4% ± 3·6	17·3% ± 4·2
ANT-2400 S10 (GQ153646)	*Pseudomonas* sp. SI85-2B AF326382 (98%)	5·7% ± 5·5	26·5% ± 1·2	28·6% ± 0·9
Gram-positive (*Actinobacteria*)				
ANT-2400 S9 (GQ153645)	*Rhodococcus* sp. A1XB1-5 AY512642.1 (97%)	40·5% ± 6·4	29·1% ± 1·9	18·4% ± 1·6
ANT-2400 S13 (GQ153649)	*Rhodococcus* sp. w-4-1 FJ544263 (99%)	62·8% ± 1·5	19·5% ± 0·6	11·9% ± 0·8
ANT-2400 S2 (GQ153638)	*Clavibacter*-like sp. V4.BE.53 AJ244680 (99%)	44·3% ± 6·2	25·0% ± 2·8	26·6% ± 2·9

strains (Hara *et al.* 2003). Our findings further confirm the widespread occurrence of *Alcanivorax*-affiliated strains in deep-sea environments (Head *et al.* 2006; Cui *et al.* 2008).

The subclass gamma *Proteobacteria* is known to harbour most aerobic HCB (van Hamme *et al.* 2003; Berthe-Corti and Hopner 2005) and in particular alkane degraders like *Pseudomonas*, *Marinobacter* and *Alcanivorax* (Gauthier *et al.* 1992; van Hamme *et al.* 2003). Our results are particularly intriguing in the sense that we have succeeded in isolating these three genuses from the same deep-sea environment in association with Gram-positive HCB.

In the case of Gram-positive isolates belonging to *Rhodococcus*, previous studies have indicated their potential capacity as HCB (Malachowsky *et al.* 1994; Sharma and Pant 2001; Aoshima *et al.* 2006; Liu *et al.* 2009). For instance, *Rhodococcus erythropolis* NTU-1 can use 50% of *n*-hexadecane (2000 ppmv) as its sole source of carbon and energy after 140 h of incubation in natural sea water (Liu *et al.* 2009). *Clavibacter* is a genus known to have several HCB strains (Gülensoy and Alvarez 1999; Dore *et al.* 2003) but until now they have not been isolated from marine environments.

Our results clearly indicated that the same active HCB group in the coastal environments may also be hydrocarbon degradation agents in the Mediterranean deep-sea sediments. In this work and in all previous studies except for that of Schwarz *et al.* (1974), all the isolates were obtained from enrichment cultures at atmospheric pressure. The isolated bacteria are certainly piezotolerant, but more studies are needed to investigate their piezobehaviour by mimicking *in situ* conditions in the deep sea (Cui *et al.* 2008), particularly the Mediterranean area. Future studies must include new isolation work in which the hydrostatic pressure corresponding to sampling depth is maintained, as we cannot exclude the presence of unknown piezophilic HCB with specific capacities in deep-sea sediments.

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