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Biodegradation of crude oil by thermophilic bacteria isolated from a volcano island

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Abstract

One-hundred and fifty different thermophilic bacteria isolated from a volcanic island were screened for detection of an alkane hydroxylase gene using degenerated primers developed to amplify genes related to the *Pseudomonas putida* and *Pseudomonas oleovorans* alkane hydroxylases. Ten isolates carrying the *alk*J gene were further characterized by 16s rDNA gene sequencing. Nine out of ten isolates were phylogenetically affiliated with *Geobacillus* species and one isolate with *Bacillus* species. These isolates were able to grow in liquid cultures with crude oil as the sole carbon source and were found to degrade long chain crude oil alkanes in a range between 46.64% and 87.68%. Results indicated that indigenous thermophilic hydrocarbon degraders of *Bacillus* and *Geobacillus* species are of special significance as they could be efficiently used for bioremediation of oil-polluted soil and composting processes.

Introduction

Bioremediation has been evaluated in several studies as an option to treat the oil pollution resulting from the spillage or leakage of crude oil and fuels in the environment (Ijah & Antai 2003; Verstraete & Top 1999; Yuste et al. 2000). Temperature plays an important role in controlling the nature and efficiency of microbial hydrocarbon degradation, which is of major significance for *in situ* bioremediation (Leahy & Colwell 1990). Degradation of long chain alkanes by mesophiles at temperatures between 25 and 28 °C has been extensively studied in contrast with biodegradation pathways in thermophiles that are not yet well characterized (Mishra et al. 2001; Sepic et al. 1996). It is recognized that enzymes from thermophiles are more resistant to physical and chemical denaturation, while another advantage of using thermophiles in bioremediation processes would be faster growth rates. Relative studies suggest that thermophilic hydrocarbon degraders of *Bacillus*, *Thermus*, *Thermococcus* and *Thermotoga* species occurring in natural high-temperature or sulfur-rich environments are of special significance as they could be efficiently used for bioremediation of oil-polluted desert soil, sediments in semi-arid climates with long hot summers and in composting processes (Feitkenhauer et al. 2003; Shimura et al. 1999).

Thermophiles growing on medium chain and long chain alkanes constitute a great biotechnology perspective and the number of characterized thermophiles with biodegradation potential has been increased during the last years (Feitkenhauer et al. 2003; Hao et al. 2004). Yet, little is known about the main alkane hydroxylase systems of the thermophilic degradation pathways. Homologues of the *Pseudomonas* genes that allow hydrocarbon degradation found in thermophilic bacteria can be

related with high biodegradation rates exhibited at high temperatures (Shimura et al. 1999; Smits et al. 1999; Wang et al. 1996).

In this study we report the isolation of indigenous thermophilic bacteria from the volcanic environment of Santorini Island. Seawater and soil habitats with high temperatures, high sulfur and iron concentrations (Hannert 2002) are expected to exhibit bacterial biodiversity of special interest. An *alk*J gene probe has been used to detect the presence of a wide-range distribution gene of *Pseudomonas* hydrocarbon catabolism in the thermophilic isolates. *Alk*J gene encodes a key aliphatic alcohol dehydrogonase, that is part of the well studied *alk* pathway which has homologues in different species and is necessary for *Pseudomonas* growth on petroleum hydrocarbons (Milcic-Terzic et al. 2001; Sei et al. 2003; Whyte et al. 1997). The role of the enzyme is further supported by the fact that it has been found to represent 10% of the total membrane proteins in *Pseudomonas* during induction by alkanes (Chen et al. 1996). Thermophiles found to be carrying the *alk*J gene were further characterized by 16s rDNA gene sequencing and grown in liquid cultures with crude oil as a sole carbon source in order to estimate their biodegradation potential.

Materials and methods

Sampling areas

Samples from volcanic soil, seawater and sediment from two bays near the active volcano of Santorini at Nea Kameni island (Santorini, Greece, 25°25′ N, 36°25′ E) Soil samples were taken from the surface layer (0–5 cm) of three locations in the active volcano of Santorini at Nea Kameni Island. Soil temperature in the study areas ranged between 85 and 95 °C. Samples were collected in sterile glass 5 l bottles.

Samples of seawater were taken from two sites in Nea and Palaea Kameni Islands. The temperature in the first site ranged between 30 and 35 °C whereas in the second site between 28 and 30 °C. For the isolation of thermophilic bacteria, sterile plastic containers were filled with 5–10 l of seawater at 80 cm below surface. Samples were stored at 4 °C and filtered on 0.22 μm filters within 24 h.

Additionally, sediment samples were obtained from Palaea Kameni site. Sediment temperature ranged between 25 and 26 °C. A plastic sterile core (diameter, 5 cm) was used to collect upper 5 cm of the sediment. Soil and sediment samples were stored at room temperature and were analyzed within 48 h.

All samples were collected at three seasonal samplings per year from May 1999 to October 2001.

Isolation of thermophilic bacteria

Soil or sediment samples (50 g) were placed in sterile Erlenmeyer flasks, mixed with 450 ml sterile Ringers solution (0.25 strength) and shaken on an orbital shaker at maximum speed (500 rev min^{-1}) for 30 min. Water samples and supernatant from soil and sediment samples mixed with Ringer solution (400–500 ml) were filtered at room temperature through filters (0.22 μm GV, Durapore®, Millipore), and the particulates collected were resuspended in 10 ml of Ringers solution. Aliquots (200 μl) of these suspensions were spread onto the surface of agar – solidified media (Table 1), and triplicate plates were incubated aerobically at 60 °C and 80 °C for 2–4 days.

Enrichment cultures were also included in the isolation procedure. For this purpose, aliquots (1 ml) of the forenamed suspensions were inoculated into nutrient-rich, nutrient-poor, and mineral salts growth media (Table 1). Triplicate of these enrichment cultures were incubated aerobically at 60 °C and 80 °C for 3–5 days. Single colony isolates were obtained from enrichment cultures by plating on all agar solidified media described in Table 1.

Table 1. Media used for isolation of thermophilic bacteria strains

Medium	Source/reference
Thermophilic *Bacillus* medium	Atlas (1993)
R2A	LAB M
NA	MERCK
Marine agar	Atlas (1993)
NB 50%	DIFCO
NB 10%	DIFCO
Mineral salts for thermophiles	Atlas (1993)

For solidified media 3% (w/v) agar was added.

The above procedures were followed as described for all different seasonal samples obtained from all the described sites.

Primer design and alkJ gene detection

Oligonucleotide primers to amplify a region of *alk*J gene were designed on the basis of published sequences by using the Hitachi Software DNASIS. PCR amplification of a 352 bp fragment of the *alk*J gene, corresponding to nucleotides 16631–16983 of *Pseudomonas putida* P1 [gi:11137521] *alk*J gene, was performed using the forward primer *alk*JF 5′-tcggcc(ct)aatttgcagtttc-3′ and the reverse *alk*JR 5′- tttacccat(ca)ctacaagtacc-3′. PCR mix of 50 μl final volume, contained 40 ng of template DNA, 50 mM KCl, 10 mM Tris–HCl (pH 9), 0.1% Triton X-100, 1 Unit of Promega *Taq* DNA Polymerase, 0.2 mM deoxynoucleotide triphosphates, 3.75 mM $MgCl_2$, 20 nM of each primer and 5% (v/v) acetamide. After a 5 min step at 94 °C, 35 cycles of amplification consisting of 1 min denaturation at 95 °C, 1 min of primers annealing at 58 °C and 2 min of primer extension at 72 °C, followed by a 10 min final extension step at 72 °C.

One fifth of the reaction volume was analyzed in a 1.2% agarose gel at 8 V cm^{-1}. The gels were stained with ethidium bromide, photographed using UV light and placed onto a vacuum apparatus (Bio-Rad). Transfers were made for 20 min with 250 mM HCl and 0.4 N NaOH at 140 Pa onto a Nylon membrane Hybond H^+.

Genomic DNA of *E. coli* strain DSM8830 carrying *Pseudomonas putida* OCT plasmid was used as template in PCR reaction with the *alk*J primers described above. The resulting PCR product was digested with *Hpa*II and *Msp*I to give a 290 bp region within the amplified area of the coding sequence of the *alk*J gene. The digested PCR product was used as a probe.

Probe was labeled with ^{32}P–dCTP by random priming, according to the vendor's instructions (GibcoBRL). The Southern blot membranes were prehybridized for 3 h in prehybridization solution at 62 °C. Hybridization was performed overnight in 20 ml hybridization solution (CHURCH) per 100 cm^2 membrane including 2.5 μl ml^{-1} labeled probe at 62 °C. Membranes were washed with 2× SSC, 0.1% SDS, four times for 15 min, followed by 0.2× SSC, 0.1% SDS for 15 min at 62 °C. Final wash was with 0.2× SSC, 0.1% SDS for 30 min at room temperature. Hybridization was detected by exposure to X-ray film.

16S rDNA sequencing and phylogenetic analysis of isolates

Isolated strains were harvested from 1 ml overnight cultures. DNA extraction was performed following the protocol of Haught et al. (1994), the region of their 16S rDNAs corresponding to nucleotides 8–1510 of the *E. coli* 16S rDNA (Lane 1991) was amplified and both DNA strands sequenced. Overlapping sequences were obtained using primers FGEO19 (5′-gaggcagcagtagggaatc-3′) and F984 (Nübel et al. 1996). Sequence data were compiled using the ARB software (www.arb-home.de) and aligned with sequences obtained from the ARB and GenBank databases Fast Aligner utility, and followed by manual aligning according to secondary structure. Analyses were performed using minimum evolution and parsimony methods implemented in PAUP* (Swofford 2003). Heuristic searches under minimum evolution criteria used 1000 random-addition replicates, followed by tree bisection-reconnection topological rearrangements. Bootstrapping under minimum evolution and parsimony criteria was done with 1000 replicates for both the archaeal and bacterial data sets. Sequences retrieved from this study have GenBank AY603070–AY603079.

Growth of isolates in liquid cultures

Liquid cultures were carried out in 250 ml Erlenmeyer flasks sealed with a Teflon^{reg} lined screw. The mineral salts culture medium used in biodegradation studies was adapted from Cho et al. (1997) and modified to contain per liter: K_2HPO_4 1.8 g, KH_2PO_4 1 g, NH_4NO_3 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, yeast extract 0.05 g, $Fe_2(SO_4)_3$ 0.01 g, $Na_2MoO_4{\cdot}2H_2O$ 0.005 g, $MnSO_4{\cdot}4H_2O$ 0.005 g, Na_2SO_4 2 g, NH_4Cl 3 g and KNO_3 1 g. 50 ml of the above medium was added directly to each flask and the pH of the fresh medium was adjusted to 7.8 with 2.5 N NaOH. After sterilization at 121 °C for 20 min, 2% (w/v) crude oil was added. After inoculation, flasks were incubated at 55 °C on an orbital shaker set to 180 rpm for 10 days. Control flasks which contained no inoculum, were incubated at the same conditions, for estimation of

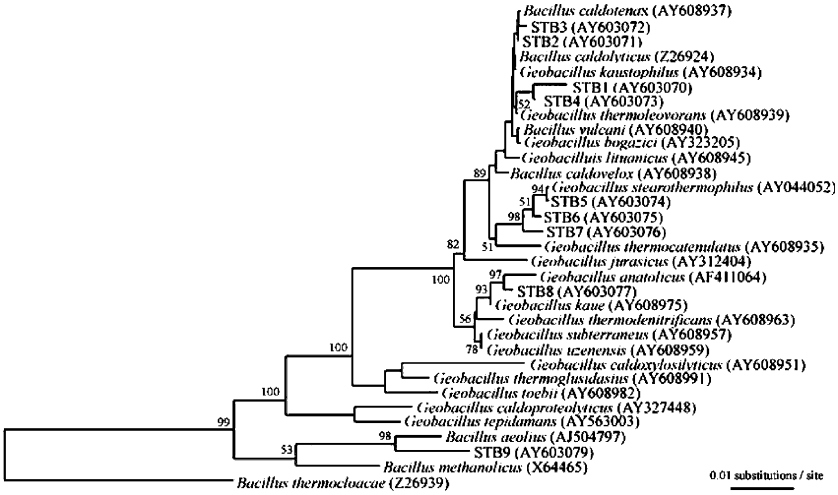

Figure 1. 16S rRNA-based minimum evolution distance tree showing the phylogenetic relationships of bacterial isolates from the volcano of Santorini Island to cultured bacterial species of *Bacillus* and *Geobacillus* species. The tree is based on *E. coli* positions 8-1510 of the 16S rRNA gene. Bootstrap (1000 replicates) support values (%) are given at nodes for minimum evolution distance. The tree was rooted with *Bacillus thermocoacae*. Note: STB10 (not shown) 16S rDNA sequence was identical to that of STB8.

abiotic loss of crude oil hydrocarbons. All experiments were performed in triplicate. Biological degradation of selected crude oil alkanes was calculated as the difference between alkane concentrations in inoculated and control samples analyzed on the same day. Results were expressed as percentage of biodegradation compared to control.

Oil extraction and biodegradation measurements by GC-MS

Cultures were extracted using equal volume of *n*-hexane. The *n*-hexane soluble fraction was analyzed by gas chromatography-mass spectrometry (GC-MS hp6890), equipped with a Saturn 4D ion trap detector, with a split or splitless injector and a fused silica column (DB5) (30 m × 0.25 mm i.d.), using He as a carrier gas. Initial oven temperature was 60 °C; temperature was increased to 290 °C at a rate of 15 °C min^{1}. The injector was set at 290 °C in the splitless mode and then changed to the split mode of 1/10 to the column. The MS had a mass range of 30–400 atomic mass units at a scan rate of 0.5 s $scan^{-1}$. The acquisition time was set for 30 min. (Figure 1)

Results and discussion

Through the enrichment and direct isolation procedures 150 thermophilic bacteria strains were obtained. Alkane hydroxylase *alk*J gene was detected in 10 of them by PCR (Figure 2) and DNA–DNA hybridization (Figure 3). Molecular

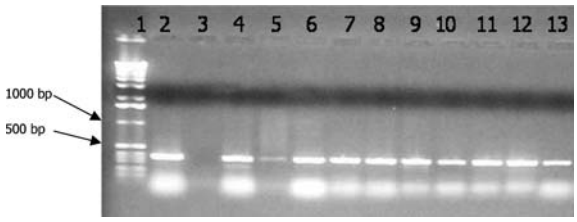

Figure 2. Detection of *alk*J gene in thermophilic bacteria. (1) 1 kb DNA ladder, (2) *E. coli* DSM8830-positive control, (4–13) isolates STB1–STB10, carrying the *alk*J gene.

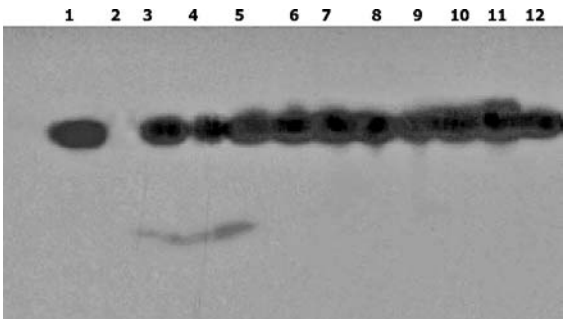

Figure 3. Detection of *alk*J gene in thermophilic bacteria using an *alk*J gene probe. (1) *E. coli* DSM8830-positive control, (3–12) isolates STB1–STB10, carrying the *alk*J gene.

detection of the *alk*J gene of *Pseudomonas* in our thermophilic isolates, under conditions of moderate stringency, implies the presence of an alkane hydroxylase gene of high homology with that found in Gram negative mesophilic bacteria. Similar results have not been reported in related studies, as only the presence of aromatic hydrocarbon degradation genes has been studied in thermophiles (Kirimura et al. 2004; Shimura et al. 1999). The occurrence of biodegradation genes in the studied strains can be related with the high biodegradation rates exhibited by the studied strains that are equivalent to biodegradation rates of bacteria widely used in bioremediation processes (Mukherji et al. 2004; Sepic et al. 1996).

Sequencing and subsequent phylogenetic analysis of the near-complete sequence of the 16S rRNA gene, identified the isolates carrying the *alk*J gene as *Bacillus* and *Geobacillus* strains (Table 2 – Figure 2). Members of *Bacillus* and *Geobacillus* have been reported to occur in oil polluted soil and petroleum reservoirs and have the ability to utilize crude oil as the sole carbon and energy source (Kuisiene et al. 2004; Orphan et al. 2000; Rahman et al. 2002; Watanabe 2001). Additionally, the ability to degrade octane and longer chain alkanes has been described as a taxonomic characteristic of *Geobacillus* genus (Nazina et al. 2001).

Despite the fact that bacteria with ability to utilize petroleum hydrocarbons occur in non polluted ecosystems only as the 8% of the total heterotroph microbial community (Leahy & Colwell 1990), the presence of *Geobacillus* is not unexpected (Schinner & Margesin 2001; Zhuang et al. 2002). The ability to form endospores is an adaption mechanism that allows bacilli to survive in intertidal marine sediments, and sediments in semi-arid climates with long hot summers that are characterized of high temperatures, osmotic pressure and non-stable or poor nutrient supply (Shimura et al. 1999).

Our data suggest that all the selected isolates were able to degrade crude oil efficiently. The total extend of degradation of crude oil aliphatic compounds ranged from 46.64 to 76.6% after 10 days for crude oil aliphatic compounds containing at least 26 carbon atoms (Table 3). For lower molecular weight aliphatic compounds (18–24 carbon atoms) degradation varied between 56.53 and 87.68% (data not shown).

These results are of the higher expected for biodegradation petroleum hydrocarbons at high temperatures (Feitkenhauer et al. 2003; Furuya et al. 2003; Ijah & Antai 2003) and support the significant biodegradation potential of bacilli that has already been described. Compared to mesophilic alkane degrading microorganisms, the measured biodegradation rates were the same or even better in the case of longer chain alkanes (Sepic et al. 1996; Vinas et al. 2002). Long chain alkanes are semi solid in lower temperatures and therefore less accessible to microorganisms growing at lower temperatures (Shimura et al. 1999; Whyte et al. 1997).

Table 2. Thermophilic bacteria isolates from Santorini volcano carrying the *alk*J gene

Isolate designation	Origin	GeneBank accession number	Sequence alignment		Nearest phylogenetic neighbour (GeneBank accession number)
			No of nucleotides[a]	% identity[b]	
STB1	Volcano soil	AY603070	1466	99.9	*Geobacillus thermoleovorans* (BT16SRRNF)
STB2	Volcano soil	AY603071	1478	99.7	*Geobacillus thermoleovorans* (BT16SRRNF)
STB3	Volcano soil	AY603072	1426	99.7	*Geobacillus thermoleovorans* (AF385083)
STB4	Volcano soil	AY603073	1349	99.8	*Geobacillus thermoleovorans* (BT16SRRNF)
STB5	Sea water	AY603074	1421	100	*Geobacillus stearothermophilus* (AY044053)
STB6	Sea water	AY603075	1417	99.9	*Geobacillus stearothermophilus* (AY044053)
STB7	Sea water	AY603076	1443	99.6	*Geobacillus stearothermophilus* (AJ586362)
STB8	Volcano soil	AY603077	1453	99.2	*Geobacillus anatolicus* (AF411064)
STB9	Sediment	AY603079	1364	99.5	*Bacillus aeolius* (BEO504797)
STB10	Volcano soil	AY603078	1453	99.2	*Geobacillus anatolicus* (AF411064)

[a]The number of 16S rDNA nucleotides used for the alignment.
[b]The percentage identity with the 16S rDNA sequence of the nearest phylogenetic neighbour.

Table 3. Percentage biodegradation of high molecular weight alkanes from crude oil after 10 days culture, compared to the uninoculated control

Isolate	Number of carbon atoms in aliphatic compound				
	C26	C28	C30	C32	C34
STB1	73.99	70.85	67.16	67.64	49.63
STB2	68.81	70.04	69.15	68.32	54.86
STB3	57.89	60.24	58.49	55.43	57.4
STB4	76.60	73.27	71.51	67.88	60.3
STB5	66.34	66.71	64.34	65.37	55.6
STB6	73.72	72.99	72.78	66.45	52.81
STB7	66.58	65.56	61.58	60.96	59.75
STB8	75.44	71.24	72.37	68.03	49.31
STB9	71.14	70.85	70.72	68.73	60.5
STB10	70.30	69.16	70.84	68.48	46.64

Conclusion

One-hundred and fifty thermophiles isolated from natural non contaminated environments, that are adapted to conditions non-supportive for microbial growth, were found to metabolize a wide range of crude oil hydrocarbons efficiently. Biodegradation rates ranging from 46.64 to 87.68% are among the higher described in bioremediation processes using thermophilic or mesophilic strains, illustrating this way, a promising biotechnology perspective. Additionally, detection of far-range biodegradation genes from mesophilic bacteria in our thermophilic isolates suggests the occurrence of horizontal gene transfer of the *alk* degradation pathway. The encouraging results of the present biodegradation studies support the unique role of thermophilic hydrocarbon degraders, as they could be successfully used in bioremediation and bioaugmentation procedures of desert or tropical soils, as well as in treatment of hot wastewater.

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