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ORIGINAL RESEARCH PAPER

A refinery sludge deposition site: presence of *nah*H and *alk*J genes and crude oil biodegradation ability of bacterial isolates

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Abstract 204 bacterial isolates from four Greek refinery sludge deposition sites were investigated for the presence of *nah*H and *alk*J genes encoding key enzymes of both aromatic and aliphatic hydrocarbon degradation pathways by PCR and DNA hybridisation. Members of *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Rhodococcus* and *Arthrobacter* play important role in bioremediation processes in sandy/loam soil contaminated with oil and *nah*H and *alk*J genes were present in the 73% of the isolates. Consortia of bacterial isolates that were used for biodegradation of aliphatic and aromatic hydrocarbons in crude oil using liquid cultures exhibited rates from 35% to 48% within 10 days of incubation.

Keywords Bacteria · Biodegradation · Genes for hydrocarbon dehydration (*nah*H and *alk*J) · Hydrocarbons

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Introduction

Oily sludges from oil refineries contain high molecular mass hydrocarbons, rich in aromatic compounds that are frequent end products of crude oil processing and are poorly degradable. The environmental problem calls for bioremediation as a potentially useful tool, based mainly on use of indigenous bacteria, in the cleaning and treatment of petroleum contaminated soils (Noncentini et al. 2000; Yuste et al. 2000).

Given the complexity of oil products and environmental conditions in a contaminated natural environment, a combination of strains with broad enzymatic capabilities is required to achieve extensive degradation (Mishra et al. 2001). Many microbial catabolic pathways responsible for the degradation, including the *nah* (naphthalene) and *alk* (*n*-alkanes) pathways have been characterised and are generally located on large, transmissible plasmids usually found in *Pseudomonas* spp. (Vinas et al. 2002). Biodegradation potentials of microbial populations from contaminated soils differ in different soil types and contamination levels, as they harbour a wide range of different degradation pathways (Vinas et al. 2002). Therefore, information of molecular ecological profile of deposition sites is useful for the development of strategies to improve bioremediation (Bundy et al. 2002).

In the present work, 204 aerobic bacterial isolates were recovered through enrichment from contaminated soil from three different refinery sludge

deposition sites with different contamination level and additionally from a non-contaminated area. Genes encoding key enzymes of both aromatic (*nah*H, encoding catechol 2,3-dioxygenase) and aliphatic (*alk*J, encoding a key aliphatic alcohol dehydrogonase) hydrocarbon degradation pathways, were detected in the above isolates by PCR and DNA hybridization using specific primers (Meyer et al. 1999; Milcic-Terzic et al. 2001; Sei et al. 2003). The isolates were further examined for their efficiency to degrade petroleum hydrocarbons and based on their biodegradation rate a group of four isolates from each sampling site was identified as a working consortium and used for biodegradation studies in liquid cultures. Occurrence of *nah*H and *alk*J biodegradation genes and biodegradation potentials were correlated to the contamination level of the soils studied.

Materials and methods

Soil

Soil samples were collected from four sites; three of them (A, B and C) were used as oily sludge disposal areas by a refinery situated 80 km from Athens, and the fourth site (D) was a non-contaminated area 1 km from the refinery plant (used as control). Each one of the three contaminated sampling sites represented different biodegradation phase: site A was sampled 6 months after the last waste disposal, whilst sites B and C were sampled 18 and 96 months after the last waste disposal, respectively. The non-contaminated area D was an agricultural area, used as control for the soil characteristics of the extended area. Soil samples were collected from 30 cm depth using a sterile 8 cm diameter plastic core. The sampling procedure followed a two dimensional matrix. The total sampling area was 20 m^2 and five soil samples were collected from each sampling area. Each sample was placed separately in new Ziploc plastic bag, transported to the laboratory, and stored at 4°C and analyzed within 3 days. Ten grams of each individual sample of every sampling site were then mixed to provide one so called "representative sample" (50 g) for the prolonged area.

The soil was classified as sandy/loam (67% sand and 10% clay) with pH of 7.2 in distilled, deionised water and moisture content of 33%.

Analysis of petroleum hydrocarbons in soil and liquid cultures

Total petroleum hydrocarbons (TPH) were extracted from 10 g soil with 100 ml each of hexane, methylene chloride and chloroform and the determination of hydrocarbon concentration at each sample was performed as described by Meintanis et al. (2006). Cultures were extracted using equal volume of each of hexane, methylene chloride and chloroform and the determination of hydrocarbon concentration at each sample was performed as described by Meintanis et al. (2006).

Selective isolation of oil hydrocarbon degraders

Bacterial strains were isolated through enrichment of the minimal salts medium (Cho et al. 1997) with crude oil 5% (w/v) or naphthalene (Fluka) 10 mM as the sole carbon source. Erlenmeyer flasks (250 ml) containing 100 ml of the above media were inoculated with 10 g soil sample, were shaken at 28°C for 48 h. Cultures were then diluted 1:1 with relevant fresh medium. The process was repeated three times analysing the cells present by serial dilution and plating in minimal salts plates supplemented with 2% (w/v) crude oil or naphthalene 10 mM. Individual colonies were inoculated in fresh media to asses their ability to degrade the carbon source provided. Crude oil [Iranian heavy crude (API gravity 30) containing 8% (w/v) asphaltenes] was added to the media after autoclave and naphthalene was provided as crystals directly placed on the plate lid or in the liquid media.

Primer design and *nah*H and *alk*J genes detection

Isolated hydrocarbon degrading strains were used for DNA extraction following the protocol of Haught et al. (1994). DNA of the isolates was next used as template in PCR for detection of the selected hydrocarbon degradation pathways.

Oligonucleotide primers to amplify regions of *nah*H and *alk*J genes encoding catechol 2,3-dioxygenase and aliphatic alcohol dehydrgonase respectively, were designed on the basis of published sequences by using the Hitachi Software DNASIS. PCR amplification of a 476 bp fragment corresponding to nucleotides 409 to 885 of *Pseudomonas putida nah*H gene [gi:45702] was performed using the forward primer nahHF 5′-GCAGACAAGGAATA(CT)ACTGG-3′ and

the reverse *nah*HR 5′-TCATGAATCGTTCGTTGA GC-3′. For the detection of *alk*J gene a 352 bp region corresponding to nucleotides 16,631 to 16,983 of *Pseudomonas putida* P1 [gi:11137521] *alk*J gene was amplified using the forward primer *alk*JF 5′-TCG GCC(CT)AATTTGCAGTTTC-3′ and the reverse *alk*JR 5′-TTTACCCAT(CA)CTACAAGTACC-3′ (Meintanis et al. 2006).

Pseudomonas putida DSM4476 carrying the NAH plasmid and *Escherichia coli* DSM8830 carrying the OCT plasmid were used as reference strains to determine the specificity of the primers designed for the detection of catechol 2,3-dioxygenase (*nah*H) and aliphatic alcohol dehydrgonase (*alk*J) genes in the isolates as described by Meintanis et al. (2006).

Gene probe preparation and hydridization was performed according to Meintanis et al. (2006).

Synthesis of bacterial consortia

All the isolates were grown in 250 ml Erlenmeyer flasks containing 50 ml mineral salts culture medium supplemented with crude oil 5% (w/v). The flasks including negative control (without inoculum), in triplicate, were incubated at 28°C with agitation 250 rpm for 10 days. Growth of the isolates was estimated by cell dry weight, as well as by total soluble cell protein measurement as described by Feitkenhauer et al. (2003). At the same time, % oil degradability in each culture was calculated as described by Jirasripongpun (2002).

Four isolates from each sampling area that combine high % oil degradability within 10 days of incubation, were selected to constitute a consortium giving four consortia A, B, C and D, according to the sampling area of origin.

16S rDNA sequencing of selected bacterial isolates

Strains confirming the consortium exhibiting the higher degradation activity were characterized by 16S rDNA sequencing. DNA extraction was performed using the MOBIO Clean Mega Prep Soil DNA Kit (MOBIO Inc., USA), the region of their 16S rDNAs corresponding to nucleotides 8–1,510 of the *Escherichia coli* 16S rDNA (Lane 1991) was amplified and both DNA strands sequenced. (GeneBank numbers of the sequences retrieved from this study are shown in Table 2 below.)

Biodegradation of crude oil by bacterial consortia

Biodegradation assays were carried out in 250 ml Erlenmeyer flasks sealed with a Teflon lined screw cap, containing 50 ml of mineral salts medium and 5% (w/v) crude oil. Each flask was inoculated with a consortium of isolates containing equal cell numbers of each isolate at 10^6 cfu ml^{-1}. Consortium cultures were incubated with agitation at 28°C for 10 days. Control flasks, which contained no inoculum, were incubated at the same conditions, for estimation of abiotic loss of crude oil hydrocarbons. All experiments were performed in triplicate. Biological degradation of high molecular weight crude oil alkanes was calculated as the difference between alkane concentrations in inoculated and control samples analyzed on the 10th day of incubation. Results were expressed as percentage of biodegradation compared to control.

Results and discussion

Physical and chemical properties of the soil samples from all sampling sites were determined. Air dried and pulverized soil was analyzed for moisture level, pH and soil particle distribution using standard methods (Supplementary Table 1). Selective isolation of hydrocarbon degrading bacteria from the oil-sludge deposition sites and the non-contaminated area resulted in a total of 204 bacterial isolates able to grow on crude oil and/or naphthalene as the sole carbon source. The number of isolates varied in the studied areas and depended on the contamination level (Table 1).

The presence of the studied *alk*J and *nah*H biodegradation genes varied among the isolates from different sampling areas. There seemed to be a relation between the percentage of isolates carrying the *alk*J gene and the contamination level of the soil (Table 1). In area C, which represented an oil deposition site at the end of the bioremediation process, the *alk*J gene was less common.

*nah*H gene involved in the aromatic hydrocarbon degradation pathway, was more common than *alk*J among the isolates from areas B and C (Table 1). Bacterial populations in areas B and C have mainly been exposed to high molecular aromatic compounds and that seemed to be reflected in their genotype. Aromatic hydrocarbons are known to be more

Table 1 Hydrocarbon concentrations in the sampling sites and number of isolates able to grow on crude oil and/or naphthalene carrying *alk*J and *nah*H genes

Sampling area	Total aliphatic hydrocarbons (mg g^{-1} soil)	Total aromatic hydrocarbons (mg g^{-1} soil)	Total number of isolates	Presence (%) of *nah*H	Presence (%) of *alk*J
A	21	2.5	13	46	77
B	17	2	84	58	37
C	12.5	2	102	31	26
D[a]	4	0.1	5	40	20

[a] Control area

resistant to biodegradation than aliphatic compounds and are often a serious problem during landfarming processes (Yuste et al. 2000; Mishra et al. 2001). As oil weathers and low molecular weight aliphatics disappear from the residual oil, bacteria possessing genes for the degradation of those hydrocarbons decline and are less important (Sotsky et al. 1994). Thus, as it is shown in Table 1, the distribution of genes for hydrocarbon degradation among the bacterial population, changes in response to the composition of the hydrocarbons in the habitat of origin (Milcic-Terzic et al. 2001).

Yet, some of the isolates from the contaminated areas (A, B and C) carried both genes for aliphatics and aromatics degradation. According to similar studies it is possible for the two degradation pathways to coexist

Table 2 Characterisation of the selected isolates comprising the consortia

Isolate designation	Hydrocarbon degradation genes		GeneBank Accession number	Sequence alignment		Nearest phylogenetic neighbour (GeneBank accession number)
	*alk*J	*nah*H		No of nucleotides[a]	% identity[b]	
MT25	+	−	DQ067199	960	97.3	*Pseudomonas oleovorans* (AF094735)
MT28	+	−	DQ067201	823	97.8	*Acinetobacter* sp. (AY167273)
MT15	+	−	DQ067202	619	98.4	*Bacillus fusiformis* (AB167231)
MT26	+	+	DQ067200	959	97.2	*Pseudomonas mendocina* (AF094734)
MT23	−	+	DQ067203	959	97.6	*Pseudomonas oleovorans* (AF094735)
MT24	+	+	DQ067204	954	98.5	*Pseudomonas mendocina* (AF094734)
MT12	+	+	DQ067205	764	98.7	*Bacillus subtilis* (AY867793)
MT19	+	−	DQ067206	817	100	*Rhodococcus opacus* (AB192964)
MT36	+	+	DQ067207	770	99.2	*Bacillus cereus* (AY987935)
MT59	−	+	DQ067208	761	97.8	*Bacillus cereus* (AY987935)
MT20	−	+	DQ067209	917	100	*Arthrobacter* sp. (AB167248)
MT96	+	+	DQ067210	882	98.9	*Pseudomonas stutzeri* (AF411854)
MT01	−	+	DQ067211	955	100	*Bacillus borotolerans* (AB199593)
MT86	−	+	DQ067212	880	100	*Bacillus cereus* (AY987935)
MT27	−	−	DQ067213	907	93.6	*Pseudomonas putida* (AY686638)
MT16	+	−	DQ067214	976	100	*Bacillus subtilis* (AY775778)

Synthesis of consortia: consortium A (MT25, MT28, MT15, MT26), consortium B (MT23, MT24, MT12, MT19), consortium C (MT36, MT59, MT20, MT96) and consortium D (MT01, MT86, MT27, MT16)

[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

(Zhang et al. 2004). Among the isolates able to grow in petroleum hydrocarbons as the sole carbon source, there were isolates carrying neither of the studied genes. This result implies the existence of biodegradation pathways in the indigenous population, with low homology to the ones studied in this work and this comment has been well established by studies on taxonomic, physiological and genetic diversity of biodegradation pathways' genes (Meyer et al. 1999).

Table 2 shows the synthesis of consortia A, B, C and D. The isolates used for those consortia belonged to genera *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Rhodococcus* and *Arthrobacter*. Table 2 also shows the presence or absence of the studied genes in each isolate.

Biodegradation rates in liquid cultures of the above consortia, using crude oil as a carbon source, were considerably higher than rates denoted in pure cultures in several studies (Table 3). Results by Mishra et al. (2001); Vinas et al. (2002), suggested that due to the complexity of crude oil as a substrate, any single bacterium could degrade at its best, if only one fraction of the compounds is present in it. Therefore, in order to achieve wide and extensive crude oil biodegradation a rather large consortium of the important species would be needed. Our data showed that, the ability of consortium A isolates, to degrade crude oil was far better than the others, maybe due to their adaptation in high hydrocarbon concentrations. Consortium D as it was expected exhibited poor degradation activity, as it contained isolates from a non-contaminated area (Tables 1, 3). Isolates belonged to the same species behaved differently in biodegradation ability probably due to their adaptation to the soil of origin (Tables 2, 3).

Table 3 Biodegradation of individual isolates and consortia

Isolate designation	% Biodegradation of crude oil's total aliphatic and aromatic hydrocarbons
MT26	31 ± 0.1
MT15	30 ± 0.1
MT28	34 ± 0.2
MT25	34 ± 0.1
Consortium A	48 ± 0.2
MT12	31 ± 0.2
MT24	35 ± 0.2
MT19	31 ± 0.1
MT23	33 ± 0.1
Consortium B	39 ± 0.1
MT36	32 ± 0.1
MT59	33 ± 0.2
MT96	33 ± 0.2
MT20	32 ± 0.1
Consortium C	35 ± 0.1
MT01	7 ± 0.1
MT27	7 ± 0.1
MT16	3 ± 0.1
MT86	3 ± 0.2
Consortium D	8 ± 0.1

Synthesis of consortia: consortium A (MT25, MT28, MT15, MT26), consortium B (MT23, MT24, MT12, MT19), consortium C (MT36, MT59, MT20, MT96) and consortium D (MT01, MT86, MT27, MT16)

Conclusion

Results of the present study suggested that there may be a relation between bacterial population shifts, presence of *alk*J and *nah*H genes involved in the pathways for aromatic and aliphatic degradation, and biodegradation potentials that occur during bioremediation procedures in the Greek sandy/loam soil. Isolates from heavily deposition sites which belonged to genera *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Rhodococcus* and *Arthrobacter* were exhibited high degradation ability and contained either one or both of the studied genes. However these isolates seemed to function even better when they constitute members of a biodegradation bacteria consortium. Contamination level enhances the changes of microbial populations that tend to adapt to the environmental conditions. This should be considered in microbial enrichment processes targeting to effective bioremediation of oil contaminated soils.

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