

Ring-hydroxylating dioxygenase (RHD) expression in a microbial community during the early response to oil pollution

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Received 24 August 2011; revised 8 November 2011; accepted 24 November 2011. Final version published online 11 January 2012.

DOI: 10.1111/j.1574-6941.2011.01270.x

Editor: Tillmann Lueders

Keywords

ring hydroxylating dioxygenases; T-RFLP; diversity: mRNA.

Abstract

The early functional response of a bacterial community from the sediments of a chronically oil-polluted retention basin located at the Etang de Berre (France) was investigated just after petroleum addition. After removing hydrocarbon compounds by natural abiotic and biotic processes, the sediments were maintained in microcosms and Vic Bilh petroleum was added. The diversity and the expression of genes encoding ring-hydroxylating dioxygenases (RHD) were examined just after the petroleum addition until 14 days focussing on the first hours following the contamination. RHD gene copy numbers and diversity were maintained throughout all the incubation period; however, transcripts were detected only during the first 2 days. One dominant RHD gene, immediately and specifically expressed in response to petroleum contamination, was related to RHD gene carried by a plasmid found in *Pseudomonas* spp. The expression of the RHD genes was correlated with high biodegradation levels observed for low molecular weight PAHs at 7 days of incubation. The study shows that the bacterial metabolism induced just after the oil input is a key stage that could determine the bacterial community structure changes. Monitoring the expression of RHD genes, key genes involved in hydrocarbon degradation, may provide useful information for managing bioremediation processes.

Introduction

Oil pollution represents the main threat for marine ecosystems. The microbial degradation is the principal mineralization process by which spilled oil is removed allowing the restoration of polluted ecosystems. Natural microbial communities modify their structure to cope with the presence of hydrocarbon compounds (Macnaughton *et al.*, 1999; Grötzschel *et al.*, 2002; Röling *et al.*, 2002; Kaufmann *et al.*, 2004). This modification process is usually preceded by a resistance stage without any observable structural change at the genomic level and, in some cases, followed by resilience (Bordenave *et al.*, 2007; Allison & Martiny, 2008). Although the resistance stage is characterized by the maintenance of the original genomic community structure, significant changes in the bacterial metabolism have been demonstrated (Bordenave *et al.*, 2007, 2009; Païssé *et al.*, 2010). As a consequence, an important part of oil compounds is degraded

during this period (Head *et al.*, 2006). The mechanisms underpinning the 'genomic' resistance period may have a key role in the following steps conducting to the reorganization of bacterial community structure. Thus, further information on the metabolic short-term response of bacterial community is still required to understand how the community deal with the presence of pollutants and perform an efficient bioremediation.

The examination of key genes involved in oil biodegradation can provide functional information on microbial processes occurring in response to crude oil contamination in the early resistance stage. The ring-hydroxylating dioxygenase genes (RHD), involved in the degradation of polyaromatic hydrocarbon compounds (Cerniglia, 1992), have been used as target genes for the description of petroleum degradation capacities in different environments (Nam *et al.*, 2001; Tuomi *et al.*, 2004; Chadhain *et al.*, 2006; Park & Crowley, 2006; Zhou *et al.*, 2006, 2008; Gomes *et al.*, 2007; Bordenave *et al.*, 2008; Cébron

et al., 2008; Lozada *et al.*, 2008). Thus, following the RHD diversity and gene expression during the short-term period following crude oil contamination will provide useful information to characterize the behaviour of hydrocarbonoclastic community during the resistance period.

The aims of this study were to (1) examine the polycyclic aromatic hydrocarbon (PAH) degradation abilities of a bacterial community during 14 days of incubation; (2) investigate the diversity of RHD genes and transcripts present in a bacterial community well adapted to hydrocarbon; and (3) profile the short-term oil contamination response by monitoring the RHD gene expression of the whole bacterial community. For these purposes, marine sediments, maintained in microcosms, were spiked with petroleum. The PAH biodegradation was examined by GC-MS. The RHD gene diversity and expression were characterized by conventional PCR assays, quantitative real-time PCR, terminal restriction fragment length polymorphism (T-RFLP) and library analyses.

Materials and methods

Microbial mat sampling

Heavy contaminated sediments were collected from a retention basin located in the Berre lagoon (43°29'05"N; 5°11'17"E). This basin, used as petrochemical plant treatment since several decades, presented high amount of polycyclic aromatic hydrocarbons trapped in sediment (Paissé *et al.*, 2008). Both subsurface sediments and the upper over-oxygenated thin cyanobacterial mat layer were sampled in October 2005 from station 1 of the retention basin that is immediately adjacent to the pollution input. A fraction of homogenized sediments (0.5 g) were directly frozen in liquid nitrogen and stored at -80 °C prior to the analysis for the determination of the RHD gene diversity. The remaining sample was maintained in cold boxes (4 °C) during transport.

Microcosm set-up

Fresh sediments were homogenized through 3-mm sieve, then incubated 4 weeks at day light and room temperature under gentle agitation in order to remove low molecular weight hydrocarbons. Sediments were then maintained in microcosm consisting of 40% wet sediments and 60% sterile synthetic sea water (SSW) at salinity of 20 practical salinity unit (KCl 0.428 g L⁻¹; CaCl₂·2H₂O 0.84 g L⁻¹; NH₄Cl 1.514 mg L⁻¹; MgSO₄·7H₂O 3.794 g L⁻¹; MgCl₂·6H₂O 3.017 g L⁻¹; NaCl 15.14 g L⁻¹; Na₂CO₃ 0.15 g L⁻¹; oligoelement solution SL12 (Overmann *et al.*, 1992) without EDTA; phosphate buffer 10⁻⁵ M) and

stabilized under agitation at 150 r.p.m. for 1 week. Then, 16 mL of sediment was distributed in 100-mL cotton-capped bottles and incubated for two more days under aerobic conditions prior to oil contamination (Paissé *et al.*, 2010).

Vic Bilh petroleum of 274 ± 28 mg (Vic Bilh oil field in South East of France; the main characteristics of Vic Bihl oil are density 926.71 kg L⁻¹ at 15 °C, viscosity 30 Cst at 50 °C, API gravity: 21) was added in half of the microcosm bottles (biotic oiled condition, B + OIL), while the remaining microcosm bottles served as biotic controls (B - OIL). An abiotic oiled control (AB + OIL) was also set up containing SSW, sterile Fontainebleau sand (VWR International) and Vic Bilh petroleum, which is the more accurate control to follow abiotic transformations. Sterile sand was washed three times (15') with dichloromethane under ultrasonic treatment before use. Microcosms were maintained 14 days at room temperature and day light. In order to prevent anoxic conditions, microcosms were maintained under 150 r.p.m. horizontally agitation.

PAH degradation assays

Aromatic hydrocarbon fractions remaining in microcosms were analysed in triplicates at the beginning (10 min), the middle (7 days) and the end of the experiment (14 days). At each sampling time and for each condition, three bottles selected randomly were directly placed to -80 °C until hydrocarbon extraction. The aromatic composition of Vic Bihl crude oil was also examined in triplicate at the beginning of the experiment in the same conditions as microcosm samples. Chemical extraction and quantification procedures were performed as previously described (Benlahcen *et al.*, 1997). The procedures were applied to the entire microcosm (both bottle and cap) in order to take into account the molecules adsorbed to the bottle glass or cap. The percentage of biodegradation was obtained by the difference between B + OIL and AB + OIL remaining PAH ratio (ratios relative to the hydrocarbon content at the beginning of the experiment).

DNA/RNA extraction and cDNA synthesis

To monitor the RHD gene diversity and expression changes occurring in response to oil contamination, microcosm sediments were collected in triplicates at 15 times (10 min, 1, 2, 3, 6, 12 and 24 h, 2, 3, 4, 5, 7, 9, 12 and 14 days), screening particularly the beginning of the experiment. Each sample was designated by the time of sampling XH (for samples between 0–24 h) or Xd (for samples between 2–14 days) followed by the kind of microcosms (B + OIL or B - OIL). At each sampling

time and for each condition (B + OIL or B – OIL), three microcosm bottles taken randomly were used as triplicate. Sediments of each microcosm bottle were totally subsampled in 2-mL tubes and centrifuged (8000 g) for 5 min at 4 °C. All pellets were immediately frozen in liquid nitrogen and conserved at –80 °C prior to DNA/RNA extraction. All molecular analyses except libraries were performed in triplicates (i.e. using a 2-mL tube of each microcosm bottle replicate).

Total nucleic acids were extracted using the FastRNA Pro Soil-Direct kit (Q-BIOgen) following manufacturer's instructions after a mechanical lyses step (three times for 1 min at 30 Hz) in a bead beater (TissueLyser; Qiagen) and without a matrix RNA purification step. Nucleic acid pellets were suspended in 100 µL sterile water and analysed in 1.2% formaldehyde (220 mM) agarose gel. All extracts were aliquoted and stored at –80 °C until further processing. The concentration of DNA and RNA extracts was calculated using the Quant-iT(TM) PicoGreen (R) ds DNA kit (Invitrogen) and Quant-iT(TM) RiboGreen (R) RNA kit (Invitrogen), respectively.

A concentration of 0.5 µg of DNase-treated RNA (Turbo DNase; Ambion) was reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase (M-MLV RT; USB Corporation) as previously described (Mills *et al.*, 2004) with minor modifications including the use of random hexamers (5 µL at 0.1 U A₂₆₀; Roche) and an incubation time of 2 h at 42 °C. The RT products were used immediately for PCR amplifications. Possible DNA contamination of RNA templates was monitored by PCR amplification of RNA aliquots without reverse transcription step. No DNA was detected in these reactions.

PCR, T-RFLP analysis, real-time qPCR and library construction

RHD genes or cDNA were amplified using primers designed by Bordenave *et al.* (2008) using a half-nested PCR, as described by Teo *et al.* (2002) to investigate the genes present in low copy number in target DNA. The final amplicon size was 387 bp. PCR inhibition by nucleic acid matrix was determined by 10-fold dilution series of nucleic acid matrices. Using the half-nested PCR approach, no inhibition was observed with the undiluted matrices, and thus undiluted matrices were used for diversity and quantification analysis.

For T-RFLP analysis, the first PCR was carried out with unlabelled FRT5A and FRT3B primers. The half-nested round was performed with the fluorescent-labelled TET-FRT6A and HEX-FRT3B primers. PCR were carried out using the *Taq* DNA polymerase (Eurobio) and touchdown programme running on a PTC-200 thermocycler as follows: initial denaturation step (5 min

at 94 °C), 10 cycles of denaturation (45 s at 94 °C), annealing (45 s from 64 to 54 °C, –1 °C per cycle) and elongation (1 min at 72 °C), 25 added cycles with annealing step of 45 s at 54 °C and a final elongation of 10 min at 72 °C. Purified PCR products (GFX PCR DNA and Gel Band Purification kit; GE Healthcare) were digested with 3 U of enzyme *Sau3AI* (Promega), *HaeII* and *HpaII* (New England Biolabs) separately. These enzymes lead to the highest diversity discrimination of known RHD sequences after *in silico* restriction using the GENOMICS expression v1.1 software. T-RFLP analysis was performed as previously described (Païssé *et al.*, 2008). Only T-RFs detected in at least two of the triplicates were considered.

RHD genes and transcripts were quantified using the Power SYBR[®] green PCR master mix (Applied Biosystem) in a Mx 3005P[™] (Stratagene) Q PCR apparatus as described earlier with minor changes including (1) the first-round PCR that was limited to 21 amplification cycles in order to be in the exponential amplification phase (Teo *et al.*, 2002), and (2) unlabelled primers that were used for the second-round PCR. The standard curve for gene number quantification was performed with the FRT5A-FRT3B PCR product cloned in the pCR2.1 Topo TA cloning kit (Invitrogen). The standard curve for the transcript quantification was performed with the RNA obtained after *in vitro* transcription of the cloned FRT5A-FRT3B PCR product, using the Megascript[®] kit (Mobio). The Ct value was calculated manually. The real-time PCR efficiency average was 99.18% (±8.9%), and the R² medium was 0.99 (±0.01). The nested PCR allows decreasing the limit of detection to 3 × 10³ and 8 × 10¹ copies g^{–1} of dry sediment for genes and transcripts, respectively. The transcript/gene ratio was determined in order to estimate the RHD gene expression level.

In order to identify the *in situ* and microcosms RHD genes or transcripts, clone libraries were constructed with unlabelled PCR products (obtained with the same primers as for T-RFLP) from DNA (*in situ* and 3H B + OIL samples) and RNA (3H B + OIL) using the pCR2.1 Topo TA cloning kit (Invitrogen) following manufacturer's instructions. Ninety-six clones per library were selected randomly, and their inserts were amplified using M13 primers (Eurogentec) surrounding the cloning site. The amplicons were analysed by RFLP with *Sau3AI* and *HpaII* in the same conditions used for T-RFLP. One clone representative of each restriction pattern was sequenced and analysed as described earlier (Païssé *et al.*, 2008). These sequence data have been submitted to the EMBL database under accession No. FM957080–FM957092. Corresponding amino acid sequence was obtained using the free FAST-PCR software version 3.8.82 (<http://primerdigital.com/fastpcr.html>).

Results

PAH removal

The sediments from the Berre lagoon retention basin were characterized by high hydrocarbon content ($96 \mu\text{g g}^{-1}$ of dried sediment), 75% of which were low molecular weight PAHs such as naphthalene, phenanthrene and their methylated forms. After 5 weeks of ageing, the PAH concentration decreased reaching $4.8 \mu\text{g g}^{-1}$ of dried sediment.

In order to study the bacterial response to petroleum addition, Vic Bihl crude oil was added to the aged sediment. This petroleum was composed of 76% of *n*-alkanes and 24% of PAHs. The Vic Bihl aromatic fraction contained mainly low molecular weight and sulphur heterocyclic PAHs (Table 1), naphthalene, phenanthrene, dibenzothiophene and their methylated forms representing nearly 99% of total PAHs. After 14 days of incubation, 77.3% ($\pm 16\%$) of the initial PAH content was eliminated in the B + OIL microcosm, while only 8.4% ($\pm 5\%$) of the initial PAH content was removed in the AB + OIL control microcosm (Table 1). Thus, an efficient PAH biodegradation was observed with a removal of 46.7% and 68.9% of the initial PAH content at 7 and 14 days of incubation, respectively. Most of the low molecular weight PAHs (2 and 3 aromatic rings), such as naphthalene, dibenzothiophene, phenanthrene, acenaphthylene, acenaphthene, fluorene and anthracene, were obviously biodegraded during the first 7 days. Their methylated forms were more resistant to biodegradation. The four-ring PAHs were not attacked during the first 7 days of incubation but were widely biodegraded during the second week of incubation (pyrene and methylchrysene). For the five-ring PAHs, only the dibenzoanthracene, dibenzochrysene and indeno[1,2,3-*c,d*]pyrene were partially biodegraded during the second week of incubation. It is likely that the unsubstituted and monomethylated low molecular weight molecules were biodegraded first during the first 7 days, followed by the degradation of their di- and trimethylated form and then by the degradation of higher molecular weight molecules.

Expression profiling and quantification of RHD genes

The expression profiling of RHD genes and the RHD mRNA quantification were performed by RHD-targeted RT-PCR assay and quantitative-RT-PCR (Q-RT-PCR), respectively. RHD genes were unexpressed in the uncontaminated slurries (B – OIL) during all the incubation period, while they were expressed in B + OIL microcosm immediately after the oil addition and until 2 days of

incubation. The RHD transcripts were detected in all B + OIL microcosm biological triplicates until 6 h of incubation; at 12 and 24 h, they were detected in two replicates and only in one replicate at 2 days of incubation. Consistently, in spite of this heterogeneity, the quantification of RHD transcripts in the B + OIL slurry by Q-RT-PCR indicated that the maximum of RHD transcripts was observed until 12 h (between 1×10^4 and 2×10^7 copies g^{-1} dry sediment). The quantification of RHD genes in the B + OIL microcosms revealed a stable amount of RHD gene copy number during the first 2 days of incubation with a mean of $1.44 \times 10^5 \pm 4.5 \times 10^4$ copies g^{-1} of dry sediment. Thus, the transcript/gene ratio revealed an increase in RHD gene expression level in response to the oil addition with a maximum of RHD gene expression level at 12 h (Fig. 1). At 24 h, although RHD transcripts were detected by RT-PCR, the transcript could not be quantified by Q-RT-PCR. The RHD gene expression observed immediately in B + OIL microcosm could be explained by the delay of 10 min occurring between oil addition and sample freezing that can allow the bacterial community to respond to the presence of contaminant.

Diversity of RHD genes and transcripts

The diversity of RHD gene expressed in B + OIL microcosm examined by T-RFLP showed only few T-RFs (Fig. 2). Similar low diversity was observed irrespective of the restriction enzyme used (the number of main T-RFs was 3, 4, 6, 6, 4 and 2 for 5'-HpaII, 3'-HpaII, 5'-Sau3AI, 3'-Sau3AI, 5'-HaeII and 3'-HaeII, respectively). It is noteworthy that the T-RF relative abundance showed low variance between microcosm triplicates (average variance between 1.6% and 5.9%, depending on terminal end and enzyme). The T-RFLP profiles were dominated by one T-RF (~ 40 –70%) corresponding to T-RF of 75, 315, 333, 49, 180 and 206 bp for 5'-HaeII, 3'-HaeII, 5'-Sau3AI, 3'-Sau3AI, 5'-HpaII and 3'-HpaII profiles, respectively. The relative abundances of the T-RFs were roughly maintained during all the incubation period (Fig. 2). This observation reinforces the hypothesis that the increase in the RHD transcripts was the consequence of the increase in the expression level of these genes as observed with the transcript/gene ratio (Fig. 1).

In order to identify the RHD sequence corresponding to the dominant T-RF expressed in response to the oil addition, a RHD cDNA library was constructed from the 3H B + OIL sample (3H mRNA B + OIL library), which was considered as representative of all samples expressing the RHD genes because its T-RFLP pattern was similar to those observed for the other samples with a balance in the low abundant T-RFs (Fig. 2). HpaII and Sau3AI RFLP analysis of 96 clones revealed two different operational

Table 1. Initial concentrations of target PAHs in Vic Bihl petroleum, cumulative remaining abundance (%) in microcosms after 7 and 14 days and efficiency of biodegradation determined at 7 and 14 days

Target PAHs	Number of aromatic ring	Proportion in Vic Bihl PAH fraction (%)	Remaining PAHs (%)				Biodegradation (%)	
			7d-AB + OIL	7d-B + OIL	14d-AB + OIL	14d-B + OIL	7 days	14 days
Naphthalene	2	75.9	61.5	1.8	36.4	1.7	60	35*
Methyl naphthalenes	2		90.7	0.7	74.6	0.4	90	74*
Dimethyl naphthalenes	2		98.7	11.5	90.9	2.0	87	89
Trimethyl naphthalenes	2		99.3	52.7	94.5	11.3	47	83
Tetramethyl naphthalenes	2		102.9	96.6	96.5	25.0	6	72
Dibenzothiophene	2	17.2	107.7	22.9	101.9	6.3	85	96
Methyl dibenzothiophene	2		101.0	76.2	98.4	24.0	25	74
Dimethyl dibenzothiophene	2		104.7	120.1	101.8	43.2	0	59
Acenaphthylene	2	0.6	92.7	11.4	85.8	2.0	81	84
Acenaphthene	2		94.1	23.9	95.6	5.1	70	91
Fluorene	2		106.4	10.5	102.6	2.5	96	100
Phenanthrene	3	57	113.6	25.8	109	9.4	88	100
Methyl phenanthrene	3		107.5	69.0	106.3	26.2	39	80
Dimethyl phenanthrene	3		107.9	106.7	104.5	43.5	1	61
Trimethyl phenanthrene	3		109.1	120.7	105.5	51.7	0	54
Fluoranthene	3	0.3	103.2	136.3	97.3	56.9	0	40
Benzo[b]naphtho[2,1,d]thiophene	3		108.1	126.4	102.3	54.5	0	48
Anthracene	3		108.3	31.9	120.9	25.4	76	96
Benzo[b]fluoranthene + benzo[k]fluoranthene	4	0.3	119.3	134.7	112.5	68.2	0	44
e+Benzo[j]fluoranthene	4		106.7	138.5	97.6	78.4	0	19
Triphenylene+chrysene	4		106.4	105.0	92.6	54.9	1	38
Methyl chrysenes	4		104.1	160.8	100.4	41.0	0	59
Pyrene	4		84.7	92.0	72.8	40.3	0	33
Benzo[a]anthracene	4		99.8	103.0	82.1	68.1	0	14
Dibenzo[a]anthracene+Dibenzo[a]chrysene	5		84.9	96.1	136.3	82.1	0	54
Indeno[1,2,3-c,d]pyrene	5		104.8	133.6	100.1	106.1	0	0
Benzo[e]pyrene	5		100.4	119.7	85.1	95.9	0	0
Benzo[a]pyrene	5		81.4	136.8	75.2	93.7	0	0
Perylene	5		98.8	52.1	91.6	22.7	46.7	68.9
Total target PAHs		100						

*Decrement in values is because of the depletion by volatilization in abiotic control at 14 days.

taxonomic units (OTUs) (RFLP profile). The sequences of 10 clones of the dominant OTU (98%) corresponded to the clone72 3H mRNA B + OIL. The minor OTU (2%) corresponded to the clone10 mRNA 3H B + OIL (Fig. 3). Both sequences were affiliated to *nahAc* dioxygenase gene sequences carried by the plasmid pDTG1 found in *Pseudomonas putida* NCIB 9816-4 (Fig. 3, cluster III). These sequences were related to the dominant T-RF of 75 bp (5'-end HaeII; Fig. 2) by HaeII *in silico* digestion and further confirmed with HpaII and Sau3AI *in silico* digestions. The other T-RFs (69, 73 and 387 bp with 5'-end HaeII; Fig. 2) could not be identified by library analysis. Nevertheless, *in silico* digestions of databank sequences related the T-RF 387 bp to sequences of the cluster II.

In order to better understand the RHD gene expression profile obtained after oil contamination, the diversity of RHD genes was examined in the retention basin sediment

(*in situ* DNA) and in the 3H B + OIL microcosm by library analyses. The *in situ* DNA library showed eight different RFLP profiles (OTUs), while three OTUs were detected in the 3H B + OIL DNA library. Phylogenetic analyses of a representative sequence for each OTU dispatched the sequences within two clusters (Fig. 3). Most of the *in situ* DNA library sequences (92.3%) were affiliated to sequences belonging to cluster I that includes *nahAc*, *pahAc*, *phnAc* and *nagAc* RHD gene sequences related to the *Burkholderia*, *Ralstonia*, *Delftia* and *Comamonas* genera. The other sequences (7.7%) of the *in situ* DNA library and all the sequences of the 3H B + OIL DNA library were affiliated to sequences of cluster II. The *in situ* DNA library sequences were related to *nahAc* and *pahAc* sequences of *Pseudomonas* and *Marinobacter* genera, whereas the sequences of the 3H B + OIL DNA library showed more than 99% similarity with the *nahAc*

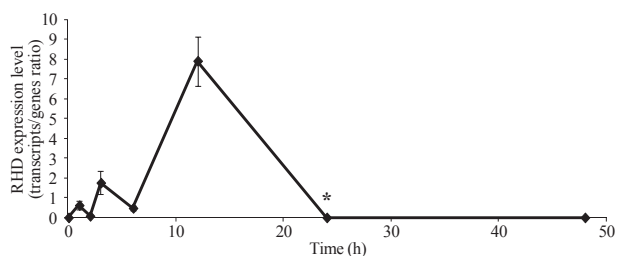


Fig. 1. RHD gene expression level. The transcript/gene ratios were calculated for the first 48 h of microcosm incubation. Each dot represents the mean of three measure replicates and three microcosm replicates (i.e. mean of nine measures). Each bar error represents the variance between the nine replicates of RHD gene quantifications. *Amplification detected by conventional RT-PCR but not quantifiable by real-time RT-PCR.

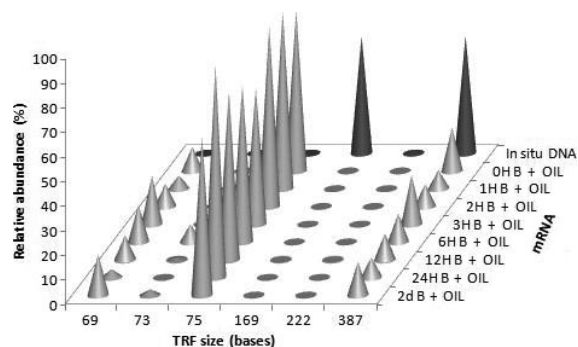


Fig. 2. Relative abundance average of T-RFs detected in microcosm triplicates and corresponding to Etang de Berre *in situ* RHD genes (in black) and mRNA RHD (in grey). The analysis presented here is based on 5'-end Haell T-RFLP patterns.

gene of *Marinobacter* genus. Thus, it is likely that the incubation conditions resulted in the selection of bacteria carrying RHD genes affiliated to the cluster II. It is important to notice that almost all the sequences of RHD gene transcripts (3H B + OIL mRNA library) fell in the cluster III with sequences retrieved in a previous study conducted in 2001 (3).

Discussion

The first stage occurring just after petroleum contamination is a key step in which the bacterial communities modify their metabolism in response to the presence of pollutant. This response would depend on several factors that include, among others, the pollution type and quantity, as well as the history of site pollution (Evans *et al.*, 2004; Bordenave *et al.*, 2007; Paissé *et al.*, 2010). Thus, the characterization of this first stage is a challenge, at academic point of view, to understand how bacterial

communities modify their metabolism to the presence of pollutant and, at applied point of view, to gain information for a better monitoring of the *in situ* bioremediation start-up processes. Developing predictive tools for the evaluation of degradation capacities that should be used as soon as possible after oil contamination is of major importance for the activation of bacteria that first respond and degrade hydrocarbons for a successful installation of bioremediation process.

The present study integrates a larger experiment designed to examine the first-stage response of bacterial community from the Etang de Berre sediments to an oil input. These sediments, exposed to petroleum contamination for several decades, have been maintained in microcosms and submitted to oil contamination. In a previous study characterizing the general bacterial communities trends in response to oil addition, we demonstrated the induction of the general metabolism few hours after oil addition resulting in a shift in the bacterial community structure the third day (Paissé *et al.*, 2010), indicating that crucial processes may occur rapidly just after the oil addition. The Vic Bihl petroleum is largely composed by *n*-alkanes, which were efficiently biodegraded [$\sim 30\%$ in 7 days and $\sim 60\%$ in 14 days (Paissé *et al.*, 2010)] with a concomitant increase in related alkanotroph transcripts (Paissé *et al.*, 2011). Similar easily utilization of *n*-alkanes by bacteria in the first stage of pollution has been previously reported (Head *et al.*, 2006; Wang *et al.*, 2010). Because alkane degradation markers were not specific enough to relate with petroleum contamination (Paissé *et al.*, 2011), PAH degradation markers (i.e. RHD genes and transcripts) were used to characterize the first-stage response of pollution. To investigate the RHD genes in the environment, many primers have been designed (Iwai *et al.*, 2011). We selected FRT primers (Bordenave *et al.*, 2008) targeting specifically gram-negative hydrocarbonoclastic bacteria (Bordenave *et al.*, 2008; Iwai *et al.*, 2011) for three reasons. First, FRT primers were already successfully applied to the Etang de Berre sediments demonstrating their specificity and their capacity to recover a large RHD gene diversity. They allow the amplification of 387 bases from the ~ 1420 bases of the complete RHD- α -subunit-encoding gene sequence (Bordenave *et al.*, 2008), and the phylogenetic tree constructed with this primer pairs is consistent with the tree obtained with the complete sequence (data not shown). The previous validation of primers is a crucial step required for each environment studied because the PCR amplification efficiency depends on the matrix (Iwai *et al.*, 2011). Second, the FRT primers target the gram-negative RHD genes-harboured bacteria that have been demonstrated to be the dominant bacteria in the Etang de Berre sediments (Villanueva *et al.*, 2004; Bordenave *et al.*, 2008). The RHD gene

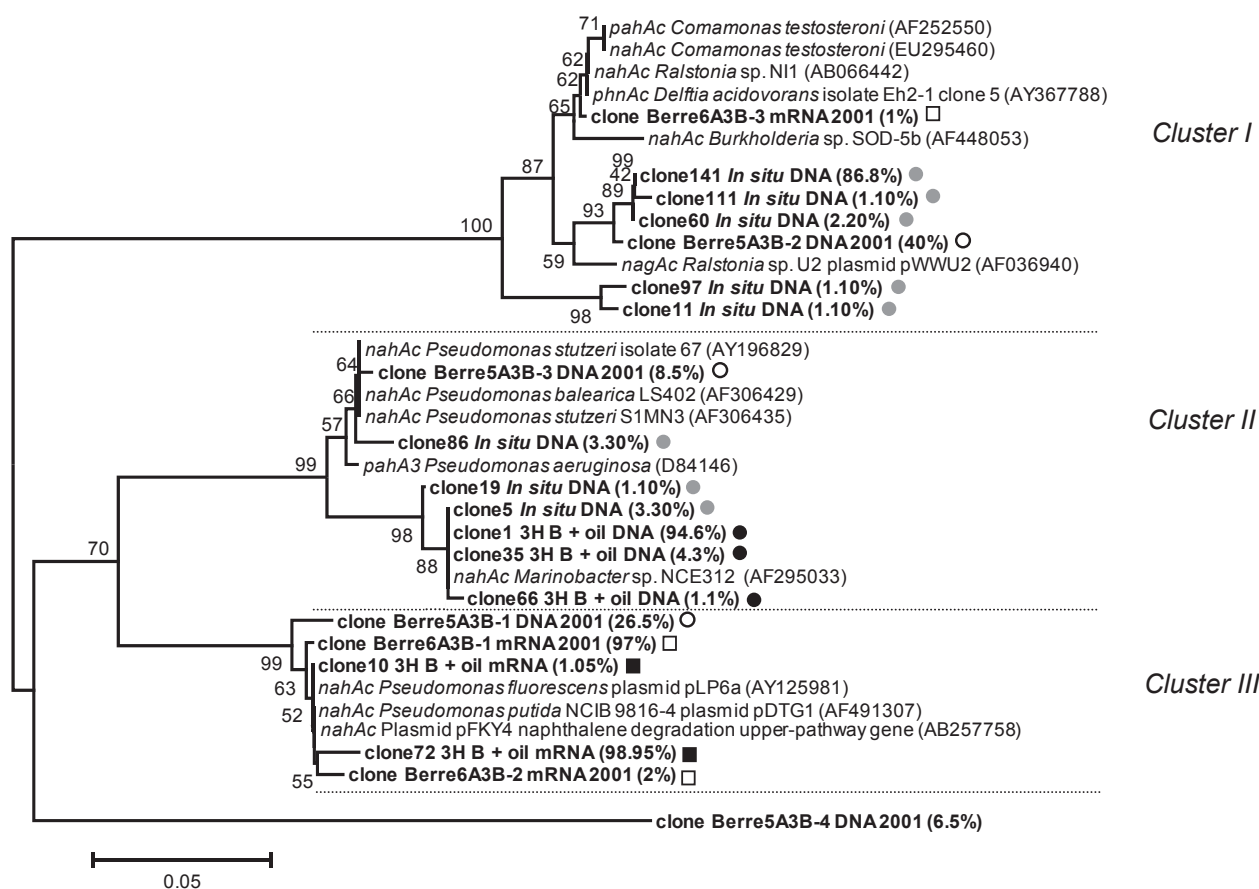


Fig. 3. Phylogenetic tree based on the analysis of the nucleotide sequences (270 bp) of amplified RHD genes from *in situ* Etang de Berre sediment (*in situ* DNA), RHD gene (3H B + OIL DNA) and RHD cDNA from slurry (3H B + OIL mRNA). All amplified products were obtained by half-nested PCR (FRT5A-FRT3B followed by FRT6A-FRT3B). The numbers in parenthesis indicate the relative abundance of clones in the corresponding library. The scale bar corresponds to 0.05 substitutions per nucleotide position. Percentages of 1000 bootstrap (above 40%) resampling that supported the branching orders in each analysis are shown above or near the relevant nodes. Symbols: (●) *in situ* DNA library, (■) 3H B + OIL DNA library, (○) 5A3B DNA 2001 library (3) and (□) 6A3B mRNA 2001 library (3).

diversity depends on the bacterial group (Gram positive or negative) as well as on the substrate specificity (biphenyl or PAH) of the RHD enzyme (Habe & Omori, 2003). The use of universal primers targeting all the genes results in unspecific amplification that impedes their use for not sequencing molecular techniques. Thus, although FRT primers are limited to gram-negative bacteria, we assume that their use provides the better diversity picture in the Etang de Berre sediments. And third, the size of the PCR product amplified with FRT primers allows to use the same primers for both diversity and quantification analyses.

Using the FRT primers, a dominant transcript related to the RHD gene carried by a plasmid found in *Pseudomonas* spp. was found expressed immediately and specifically in response to the oil addition. This gene was expressed only during the first 2 days after oil addition

with the concomitant degradation of low molecular weight PAHs observed at 7 days of incubation. The time occurring between the gene induction by a pollutant and the degradation of the compound is still under discussion. Long and stable expression of *nahAc* gene has been observed in mangrove sediment slurry contaminated with three PAHs which was associated with the biodegradation of 3- and 4-ring PAHs in 5 and 15–20 days, respectively (Zhou *et al.*, 2008) but the applied PAH concentrations were 1000-fold higher than those used in the present study. In our study, although the participation of other genes involved in PAH degradation cannot be excluded, we assume that the low concentration of PAHs remaining at 7 days (about 52.1% with mainly high molecular weight PAHs) could result in a RHD gene expression level under the detection limits of the PCR, explaining the fact that the RHD gene expression was detected only

during the first 2 days after oil addition. The detection limit has been established around 10^4 and 10^5 cells g^{-1} of soil using real-time PCR (Jacobsen & Holben, 2007; Nicolaisen *et al.*, 2008). On the other hand, the fact that high molecular weight or substituted PAHs were the main remaining PAHs at 7 days suggested that their biodegradation might involve different genes than those targeted in this study, which are involved in low molecular weight PAH degradation (Bordenave *et al.*, 2008). This observation was consistent with the sequential degradation of PAH observed by chemical analysis and with a previous study reporting that different types of genes were expressed according to the type of PAH molecules (Zhou *et al.*, 2008). Furthermore, the changes observed in the metabolically active community structure after 2 days (Paissé *et al.*, 2010) supported the hypothesis that a shift in the hydrocarbonoclastic community occurred. Anyway, the investigation into the metabolic part of the community as a whole either by analysing the 16S rRNA transcripts (Paissé *et al.*, 2010) or more specifically by targeting the RHD-harboring bacteria revealed an immediate and specific response. The response observed within the first 2 days after oil addition is followed by a modification of the metabolic active community structure (Paissé *et al.*, 2010) and materialized, at the end of the experiment, by the increase in the relative abundance of hydrocarbonoclastic bacteria isolated from the oiled slurry (Paissé *et al.*, 2010).

The dominant RHD genes detected in the slurries were affiliated to RHD genes harboured by *Pseudomonas* and *Marinobacter* species. In the sediment of the Etang de Berre, these genes were detected codominant with those related to RHD genes from *Ralstonia*, *Comamonas* and *Delftia* species. Thus, the experiment settings reduce inevitably the gene diversity (Grötzschel *et al.*, 2002). This observation may probably be explained by the modification of bacterial community structure as previously reported (Chadhain *et al.*, 2006). The dominant transcript detected in the slurries was undetected in the metagenome, indicating that the expressed genes were not dominant in the bacterial community, while the dominant genes were not expressed. Although the microcosms set-up resulted in the selection of an hydrocarbonoclastic community, the dominant transcript was the same than that identified in the native sediments of the Etang de Berre 4 years earlier (Bordenave *et al.*, 2008) even if different procedures for nucleic acid extraction and PCR amplification were applied. The expression of RHD gene was highly maintained over time, suggesting that important PAH contamination may result in maintaining the specific RHD gene populations within a microbial community for a long period and that these populations, even minor, should have a

key role in the response to petroleum input. This transcript was related to a RHD sequence carried by a plasmid that may allow its fast dissemination by horizontal transfer, which has been demonstrated to be the most efficient mechanism of adaptation to environmental changes (Phale *et al.*, 2007). Plasmids involved in the degradation of some PAHs such as naphthalene and phenanthrene have been described (Dennis & Zylstra, 2004; Li *et al.*, 2004). Further studies with plasmid-specific markers will be required to understand the role of plasmids in the response of bacterial communities to oil input.

In conclusion, we demonstrate here that the first response of bacterial communities is crucial step in determining bacterial degradation capacities. Managing this period just after the oil spill by favouring bacteria that first respond and degrade hydrocarbon is the key for the optimization of bioremediation process. Further studies monitoring the functional changes occurring during this first event are required to fully understand the bacterial mechanisms involved in the response to the presence of crude oil.

Acknowledgements

This work was funded by the European Community Project FACEiT [(STREP-grant No. 018391 (GOCE)]. We would like to thank all partners of the FACEiT project for their useful discussions. We acknowledge the financial support by the Aquitaine Regional Government Council (France), the Ministère de l'Ecologie et du Développement Durable (MEDD – PNETOX project No. CV04000147) and the ANR (DHYVA project, No. 06SEST09).

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