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Are Alkane hydroxylase genes (*alkB*) relevant to assess petroleum bioremediation processes in chronically polluted coastal sediments?

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ABSTRACT

The diversity of alkB-related alkane hydroxylase sequences and the relationship between alkB gene expres- sion and the hydrocarbon contamination level have been investigated in the chronically polluted Etang-de-Berre sediments. For this purpose, these sediments were maintained in microcosms and submitted to a controlled oil input miming an oil spill. New degenerated PCR primers targeting alkB-related alkane hydroxylase sequences were designed to explore the diversity and the expression of these genes using terminal restriction fragment length polymorphism fingerprinting and gene library analyses. Induction of alkB genes was detected immediately after oil addition and their expression detected only during 2 days, although the n-alkane degradation was observed throughout the 14 days of incubation. The alkB gene expression within triplicate microcosms was heterogeneous probably due to the low level of alkB transcripts. Moreover, the alkB gene expression of dominant OTUs has been observed in unoiled microcosms indicating that the expression of this gene cannot be directly related to the oil contamination. Although the dominant alkB genes and transcripts detected were closely related to the alkB of Marinobacter aquaeolei isolated from an oil-producing well, and to alkB genes related to the obligate alkanotroph Alcanivorax borkumensis, no clear relationship between the oil contamination and the expression of the alkB genes could be established. This finding suggests that in such coastal environments, alkB gene expression is not a function relevant enough to monitor bacterial response to oil contamination.

Keywords Alkane hydroxylase · Gene expression · Oil contamination · T-RFLP · alkB diversity

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Introduction

Aliphatic alkanes are produced by geochemical processes as a consequence of vegetal material decay (Hornafius et al. 1999; Seewald 2003) and by living organism activities throughout the biosphere (Widdel and Rabus 2001). Besides, human processes involving petroleum extraction, transport, or processing lead to release extensively alkanes in the environment. Thus, most of terrestrial or aquatic bacteria fit out systems to cope with alkanes (Sariaslani and Omer 1992; van Beilen et al. 2001) where even some bacterial species have been characterized as obligate alkanotrophs such as Alcanivorax borkumensis and Thalassolituus oleivorans (Yakimov et al. 1998, 2004).

Several key enzymes have been identified in the bacterial alkane oxidation processes including alkane hydroxylase such as cytochrome P450 (Sariaslani and Omer 1992) and AlkB (van Beilen et al. 1994), dioxygenases (Maeng et al. 1996) or methane monooxygenases (Lieberman and Rosenzweig 2004). AlkB alkane hydroxylase systems, first described in Pseudomonas putida GPo1 (Kok et al. 1989; van Beilen et al. 1994), are highly widespread in nature with more than 250 AlkB homologues identified in at least 45 bacterial species (Smits et al. 1999; van Beilen et al. 2002, 2003; van Beilen and Funhoff 2007; Wang et al. 2010a). Some genera such as Rhodococcus, Alcanivorax, and Pseudomonas have been described carrying multiple, quite divergent, alkB genes, suggesting that each gene product could be respon- sible for the oxidation of different alkane molecules in the same strain (van Beilen et al. 2003; van Beilen and Funhoff 2007). Thus, alkB genes have been considered as bacterial functional markers to monitor the bioremediation capacities of petroleum-polluted environments. Many studies described presence, dispersion, and diversity of alkB genes in natural environment or in oil-degrading bacteria isolated from aquatic ecosystems to assess the hydrocarbon degradation capabilities of natural bacteria (Kuhn et al. 2009; Hamamura et al. 2008; Heiss-Blanquet et al. 2005; Sei et al. 2003; Wang et al. 2010a, b). However, besides

petroleum compounds, algae, plants as well as other non-biogenic resources contribute to the alkane input in natural aquatic environ- ments. Thus, whether alkB gene dispersion, diversity, and expression within complex bacterial communities could be associated specifically to petroleum contamination or to the whole hydrocarbon contents of their environment remains unclear.

Polymerase chain reaction (PCR) primers and DNA probes targeting alkB genes have been developed (Whyte et al. 1996; Smits et al. 1999; Kloos et al. 2006) to detect alkB genotypes from various environments. However, the lack of specificity of these probes does not allow the screening of alkB diversity within complex ecosystems by fingerprint molecular techniques (Vomberg and Klinner 2000; Whyte et al. 1996, 2002). As well, several primers were designed to target specific lineages of alkB genes including known alkane-oxidizing Pseudomonas spp., Acinetobacter spp., and Rhodococcus (Hamamura et al. 2008; Heiss-Blanquet et al. 2005). Nevertheless, they restrict the investigation to the targeted bacterial groups. In the current study, new primers were designed in order to detect a broader range of alkB lineages, enhancing primer specificity to the known phylogenetically distinct alkB sequences. These new degenerated primers were used to: (1) investigate the diversity of the alkB genes inhabiting the long-term oil- contaminated Etang-de-Berre sediments and (2) character- ize their expression when submitted to a control oil input (performed on microcosm experiments). Because a gene marker should respond specifically to the targeted pollutant (here, petroleum-derived aliphatic hydrocarbons), we ex- amined the alkB gene expression during the early stage after the contamination, when the physiological adaptation of the community's members occurs (Païssé et al. 2010). The expression of the whole alkane hydroxylase gene pool was followed by fingerprinting method (terminal restriction fragment length polymorphism (T-RFLP)) and cDNA library analysis. Exploring the alkane hydroxylase gene diversity and expression capacity occurring in response to an oil input will

help to improve our knowledge on the ecosystem functioning.

Material and methods

Microbial mat sampling and microcosm set-up

Contaminated subsurface sediments were collected from a retention basin located in the Berre lagoon collecting hydrocarbon-contaminated water from a petrochemical industry (43°29'05''N; 5°11'17''E). This basin, used as a plant treatment since several decades, presented high amount of hydrocarbons trapped in sediment (Païssé et al. 2008). Subsurface sediments were sampled in October 2005 from station 1 of the retention basin which is immediately adjacent to the pollution input (Païssé et al. 2008). A fraction of homogenized sediments (0.5 g) were directly frozen in liquid nitrogen and stored at −80 °C prior to in situ diversity analysis of the alkB hydroxylase genes.

Fresh sediments were placed in microcosms as previously described (Païssé et al. 2010). Briefly, after a weathering step of 5 weeks, 40% of wet sediments were incubated in sterile synthetic water (SSW) at salinity of 20 PSU with (274± 28 mg) or without Vic Bilh petroleum constituting respec- tively the biotic oiled condition, (B+OIL) and the biotic control (B−OIL). An abiotic oiled control was also set up containing SSW, sterile Fontainebleau sand, and Vic Bilh petroleum. Triplicate microcosms were maintained 14 days under 150 rpm horizontal agitation at room temperature and day light (Païssé et al. 2010). Slurry sediments were collectedat0,1,2,3,6,12,and24handthen2,3,4,5, 7, 9, 12, and 14 days. For each sampling, three microcosms were sacrificed for microbiological analyses.

In situ and microcosm hydrocarbon compounds analysis

The aliphatic fraction remaining in microcosms was analyzed in triplicates at the beginning (0 hour), the middle (7 days) and the end of the experiment (14 days). The microcosms were directly placed to -80°C for hydrocarbon extraction. Briefly, sediment samples (2 g) were chemically dried with 2 g of anhydrous sodium sulfate (Na_2SO_4) and extracted with 6 mL of hexane : dichloromethane (1 : 1). After centrifugation (5897 *g* for 20 min), extracts were cleaned on SupelcleanTM solid-phase extraction (SPE) tubes as recommended by the manufacturer (SPE Supelclean EnviTM-18, Supelco Bellefonte). Extracts were diluted 10 times for GC analysis. Aliphatic hydrocarbons were identified and quantified with GC-MS using a ThermoTrace GC gas chromatograph coupled with a Thermo Trace DSQs mass spectrometer as described previously (Païssé *et al.*, 2008). The recovery percentage of the extraction method used for sediment was 85%. The variation of the reproducibility of extraction and quantification of samples were determined by successive extractions and injections (n = 6) of the same sample and estimated to be ~8%.

DNA/RNA extraction and cDNA synthesis

2 ml of slurry were subsampled and then centrifuged (8 000 g) for 5 min at 4°C. The pellet was immediately frozen in liquid nitrogen and conserved at -80°C prior to DNA/RNA extraction. Total nucleic acid were extracted from frozen pellets using the FastRNA Pro Soil-Direct kit (Q-Biogen) following manufacturer's instructions after a first step of lysis (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 agarose gel. All extracts were aliquoted and stored at -80°C until further processing. All molecular analyses except libraries were performed in triplicates.

0.5 µg of DNase-treated RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV RT, USB Corporation) as previously described (Païssé *et al.*, in press). RT products were used immediately for PCR amplifications and the remaining products were stored at -20°C. Possible DNA contamination of RNA templates was monitored by PCR amplification of aliquots of RNA without reverse transcription step. No DNA was detected in these reactions.

Design of PCR primers

Sets of primers were designed in order to amplify specifically alkane hydroxylase sequences by PCR from complex environmental sample. Degenerated primers were manually designed. A first alignment of 42 translated alkane hydroxylase sequences was performed to identify the conserved amino acid regions. alkB and alkM sequences used for the alignment belong to the four groups of alkane hydroxylase described by Heiss-Blanquet *et al.* (2005): Rhodococcus group which includes species belonging to *Rhodococcus, Burkholderia, Nocardioides, Prauserella* and *Mycobacterium* (accession number sequences: AJ301866, AJ301867, AJ301868, AJ297269, AJ401611, AJ300339, Z95121, AJ300338, AJ301870, AJ301871, AJ009586, AJ301873, AJ301869, AJ301877, AJ301876, AJ301875, AJ301874, AF350429, AJ009587, AJ293344, AJ293306), Pseudomonas group 1, including some species of *Pseudomonas, Marinobacter, Alcanivorax* and *Thalassolituus* genera (accession number sequences: CP000514, AJ233397, AJ250560, AY034587, AJ245436, AJ295164, AJ431700), Pseudomonas group 2 including other *Pseudomonas* species (accession number sequences: AJ009579, AF090329, AJ311787, AJ311786, AJ009581, AJ009580), and the Acinetobacter group which includes only *Acinetobacter* strains (accession number sequences: AB049411, AJ009582, AJ002316, AB049410, AJ009584, J233398, AJ009585). A second nucleotide

alignment of the same sequences was done to define the degeneration level of designed primers.

Three forward (AlkB1F, 5'-CAYGARYTGGGYCAYAAR-3'; AlkB2F, 5'-CAYGARYTGGGYCAYAA-3'; AlkB3F, 5'-TAYGGNCAYTTCTWYRTYGAGCA-3' and three reverse (AlkB1R, 5'-AACTAYNTYGARCAYTACGG-3'; AlkB2R, 5'-CAYTCSGAYCAYCAYGCGAATYC-3'; AlkB3R, 5'-GAYCAYCAYGCGAATYC-3') primers were designed.

PCR, T-RFLP analysis and library construction

PCR using different primer combinations was carried out using genomic DNA extract of the alkane-degrading strain *Marinobacter hydrocarbonoclasticus* SP17 (ATCC 49840). PCR (50 µl) was performed by using a reaction mixture of 200 µM each dNTP, 1.5 mM $MgCl_2$, 1 U of Taq polymerase (Eurobio), 1X PCR Buffer (Eurobio) and 0.2 µM each primer. The amplification was performed in a PTC-200 thermocycler as follow: initial denaturing step (5 min at 94°C), 35 cycles including denaturing (45s at 94°C), annealing (45s at independent tested temperature from 50 to 60°C) and elongation (1 min at 72°C), and a final elongation of 10 min at 72°C. Primer sets giving specific amplifications were then used to amplify genomic DNA from Etang-de-Berre sediment samples. As no amplification was obtained, a nested-PCR was developed in order to explore the diversity and the expression of alkane hydroxylase gene in *in situ* and microcosm samples. A touchdown PCR program was necessary at the first round of amplification using AlkB3F-AlkB3R primer set to obtain specific and high intensity amplicons at the second round of PCR. Touchdown program was performed as follow: initial denaturing step (5 min at 94°C), 10 cycles of touchdown including denaturing (45s at 94°C), annealing (45s from 60°C to 50°C, -1°C per cycle) and elongation (1 min at 72°C), 25 more cycles with annealing step of 45s at 50°C, and a final elongation of 10 min at 72°C. The

second round of amplification was done using the primer set AlkB3F-AlkB2R in the same conditions without touchdown gradient and at 50°C of an annealing temperature. For T-RFLP analysis, labelled primers were used in the second round of amplification (AlkB3F-FAM and AlkB2R-HEX). T-RFLP was carried out as previously described (Païssé *et al.,* 2008) using *Sau*3AI (Promega) and *Ava*II (New England Biolabs) separately. These enzymes were chosen after *in silico* enzymatic restriction (Genamics expression v1.1 software) of alkane hydroxylase sequences available in the databanks.

Clone libraries were carried out with unlabelled PCR products from *in situ* DNA and 6H B+OIL cDNA samples and cloned in *Escherichia coli* TOP10F' (Invitrogen Inc.) using the pCR2.1 Topo TA cloning kit (Invitrogen Inc.). 86 and 44 clones were selected randomly and their inserts were amplified using M13 primers (Eurogentec) surrounding the cloning site. The amplicons were analyzed by RFLP (Restriction Fragment Length Polymorphism) with *Sau*3AI and *Ava*II in the same conditions used for T-RFLP. One clone representative of each profile was sequenced and the phylogenetic analysis was conducted as previously described by Païssé *et al.* (2008). Corresponding amino acid sequence was obtained by using the free FastPCR software version 3.8.82 (www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm). The CHAO1 non-parametric estimator of richness was calculated considering that sequences with > 82% identity (cut-off value) assigned to the same OPF (Schloss and Handelsman, 2008).

Results

Hydrocarbon compounds distribution

The initial total petroleum hydrocarbons (TPH) concentration in the B-oil slurries (in-situ condition) was 5 mg kg^{-1}d.w sediment and the, aliphatic fraction was only representing 3% of the TPH. In contrast, the aliphatic fraction in the spiked Vic Bihl crude oil slurries (B+oil) was dominant (75% of TPH) with mainly *n*-alkanes ranging from C_{10} to C_{20} (90% of the

aliphatic fraction) had (Païssé *et al*., 2010). After 7 and 14 days of incubation, the total extractable alkanes in B+OIL slurries were reduced to 31 and 58% respectively of their initial amount.

Design of alkane hydroxylase primer set

In order to explore the diversity and the expression of alkane hydroxylase genes in the slurries, new primers were designed since the available primers (Smits *et al*., 1999; Whyte *et al*., 1996) were unable to amplify *alkB* gene either from *Marinobacter hydrocarbonoclasticus* SP17 genome or the Etang-de-Berre sediment metagenome.

The amino acid sequence alignment of 42 alkane hydroxylases showed distinct conserved regions including three histidine residues and NYXEHYG[L/M] motifs. Four of the six primers (AlkB1F, AllkB2F, AlkB2R, AlkB3R) were designed from amino acid region previously selected by Smits *et al*. (1999) and Whyte *et al*. (1996). The length and the degeneracy of these primers were reexamined to deal between diversity and specificity in order to amplify *alkB* sequences (Figure 1). All the primer set combinations (including the already available and the newly designed) were tested by direct PCR amplification using DNA from *Marinobacter hydrocarbonoclasticus* SP17 (Table 1). Specific amplification was obtained when using AlkB1F-AlkB1R, AlkB2F-AlkB1R, AlkB3F-AlkB1R, AlkB3F-AlkB2R and AlkB3F-AlkB3R primer pairs. Nevertheless, no amplification could be obtained when using these primer pairs with DNA from complex environmental matrix such as Etang-de-Berre sediment. In order to amplify *alk* gene from these sediments and slurries, a nested PCR was applied.

While most of the combinations gave good amplification, the primer set AlkB3F-Alk3R combined with AlkB3F-Alk2R gave the most efficient amplification and were used for the diversity and expression analysis. These primers provide a final amplification product of 477 bp (Table 1).

Alkane hydroxylase genes inhabiting oil contaminated sediments

The diversity of alkane hydroxylase genes was analyzed in sediments of oil retention basin from Etang-de-Berre. RFLP analysis of 88 clones identified 26 profiles. Sequence analysis of one representative clone sequence from each profile defined 7 Operational Protein Families (OPF) based on the cut-off of 18% difference between sequences (Figure 3). No unspecific amplification was observed. Coverage estimator evaluated at 9 ± 2 the richness diversity of alkane hydroxylase within this sediment. All the AlkB sequences detected in these sediments were related to sequences belonging to *Gamma-* and *Alphaproteobacteria.* 79% of alkane hydroxylase sequences were related to cluster A (Figure 3) and presented high identity with the AlkB sequence of *Marinobacter aquaeolei* VT8. This dominant OPF matched within the group *Pseudomonas* 1 defined by Heiss-Blanquet *et al.* (2005). 14% of the library presented high identity with AlkB of *Oceanicaulis alexandrii* and *Kondriimonas gwangyangensis* (cluster B). Clones affiliated to AlkB of *Parvularcula bermudensis*, *Moritella* sp., or to uncultured bacteria were represented by one or two sequences (clusters C to J) and could not be associated to any of the four groups defined by Heiss-Blanquet *et al.* (2005). Moreover, these sequences presented low sequence homology (less than 70%) with known sequences (Figure 3).

Alkane hydroxylase genes expressed in oil contaminated slurry

The alkane hydroxylase genes expressed in microcosm experiment in response to crude oil contamination was examined by T-RFLP and clone library analyses. The detection of transcripts by nested PCR indicated that the *alkB* genes were expressed immediately after the addition of oil in the B+OIL condition until 2 days (Table 2). Nevertheless, the expression

was never detected in all three replicates. The absence or the low level of expression might be explained by the absence of *alkB* transcripts detection in some replicates during these two days. The *alkB* expression was occasionally detected in the unoiled control.

T-RFLP analysis allowed to distinguish between 3 and 5 dominant OTUs depending on the T-RF end analyzed and the restriction enzyme used. The main expressed OTUs were nearly the same for all the samples (oiled and unoiled condition) showing that *alkB* expression was not strictly representative of oil spike (Figure 2). Nevertheless, the number of OTUs in oiled condition was higher than within unoiled slurries (Figure 2). DNA analysis of the Etang de Berre sediments showed higher number of OTUs than those expressed in the slurries but, in most of the cases, the dominant OTUs were identical (Figure 2). In order to identify the dominant OTUs expressed, 44 clones from *alkB* clone library of the 6h B+OIL sample were analyzed by RFLP. The phylogenetic analysis of amino acid sequences indicated that all the sequences detected expressed in slurry were affiliated to the clusters A and B, the same groups found dominant in the Etang de Berre sediment (Figure 3). 65.9% of the 6h B+OIL clones analyzed were related to AlkB of *Marinobacter aquaeolei* VT8, 25% to *Oceanicaulis alexandrii* and *Kordriimonas gwangyangensis* and 9.1% to *Alcanivorax borkumensis*. Whereas sequences of AlkB 6h B+OIL related to *Marinobacter aquaeolei*, *Oceanicaulis alexandrii* and *Kordriimonas gwangyangensis* were also detected in sediments, sequences related to *Alcanivorax borkumensis* were not. The *in silico* digestion of these sequences and the comparison with T-RFLP data confirmed the high dominance of bacteria showing *alkB* related to *Marinobacter aquaeolei, Oceanicaulis alexandrii* and *Kordriimonas gwangyangensis*. Nevertheless, OTUs related to *Alcanivorax* sequences could not be identified in the T-RFLP analysis suggesting that they were expressed at a lower level.

Discussion

In this study, we have developed degenerate PCR primers targeting *alkB*-related alkane hydroxylase sequences in order to explore the presence, the diversity and the expression of these genes in complex bacterial communities. Many primer sets were described for the amplification of *alkB* genes, targeting either the whole (Smits *et al*., 1999; Whyte *et al*., 1996) or specific bacterial groups (Hamamura *et al.,* 2008; Heiss-Blanquet *et al.,* 2005; Sei *et al.*, 2003). Nevertheless, as already observed (Vomberg and Klinner, 2000; Whyte *et al*., 1996; Whyte *et al*., 2002), the general primers led to unspecific amplification with our samples, and thus, were unsuitable for use in molecular fingerprinting techniques. The group-specific primers did not show this bias, but the number of primers sets to be used (between 3 and 9, depending on the authors) highly increases the cost and the time of the analysis. A balance between high level of degeneracy and high specificity had to be obtained for efficient primers. The newly designed primers allowed to amplify specifically *alkB* gene from Etang-de-Berre oil contaminated sediments and the PCR products were suitable for T-RFLP analysis.

Using our primer sets, most of the detected genes and transcripts belonged to AlkB *Pseudomonas* 1 group described by Heiss-Blanquet *et al*. (2005). Genes related to this group are frequently found abundant in soils and soils microcosms (Heiss-Blanquet *et al.,* 2005; Hamamura *et al.,* 2008) and were dominant in sea water microcosms contaminated with crude oil (Sei *et al.,* 2003). By the MPN-PCR techniques, Sei and coll. (2003) found that the number of copies of *Pseudomonas* group *alkB* genes were between 2 and 5 log higher than the other *alkB* groups. Considering that the sediments of Berre lagoon are mainly inhabited by gram-negative bacteria (Navarrete *et al.,* 2004), it is not surprising that the other *alkB* groups could not be detected. Moreover, even it is usually conceded that the experimental setting leads in a selection of some bacterial populations (Grötszchel *et al.,* 2002; Païssé *et al*, 2010),

the expression of the main *alkB* genes carried by the *in situ* bacteria within slurry indicate that the *alkB* containing bacteria were not submit to method selection pressure.

The expression of *alkB* genes started immediately after the oil spike to the slurries and persisted during two days, even though *n*-alkanes degradation persisted during the 14 days of incubation. Indeed, more than 65 % of aliphatic compounds remained at 7 days of incubation. Moreover, the expression was heterogeneous. *alkB* expression was never detected in the three replicates of the same sample suggesting that the expression level of *alkB* was probably near and sometimes below the detection limit. This observation suggests that the *n*-alkane degradation may involve the expression of other genes besides *alkB*. Moreover, we also detected *alkB* expression in uncontaminated slurries, suggesting that the presence and/or the expression of this gene cannot be directly related to the spiked pollution. The broad substrate specificity of *alk* gene products (e.g. biogenic alkanes) may explain the lack of specificity in the contamination response. Vic Bihl petroleum is mainly composed by n-alkanes, but there are many other alkanes in the Etang de Berre sediments originating from other sources including those produced by living organism activities throughout the biosphere (Widdel and Rabbus, 2001). Some microorganisms (plant, algae, bacteria) are able to synthesize medium and long-chain *n*-alcanes (Han and Calvin, 1969) leading to significant increase in *n*-alkanes concentration in aquatic environments (Berdié *et al*., 1995). For example, the contributions from terrestrial vegetation, particularly *Sphagnum* moss, appear to dominate the distribution of *n*-alkanes in waters of a dystrophic lake (Berdié *et al.*, 1995). With regard to biogenic inputs of organic matter, terrestrial biogenic sources seem generally to predominate in the Etang de Berre lagoon sediments according to the observed n-alkane to isoprenoid abundance ratios (Jacquot *et al.*, 1999). Moreover, previous studies detecting the *alkB* genes in both pristine and polluted environments showed the difficulties to associate the presence of *alkB* gene detection with the capacity of alkane removal. Kuhn *et al.* (2009) also found higher *alkB*

diversity in pristine environments than in contaminated. In the other hand, Palmroth *et al*. (2007) could not detect the presence of this gene in oil contaminated soils suggesting that other genes may be involved in alkane degradation. Indeed, a double mutant of *Alcanivorax borkumensis* SK12 defective in both alkB1 and alkB2 was still able to grow on medium-chain n-alkanes, indicating that genes other than *alkB1* and *alkB2* are also involved in n-alkane hydroxylation (Hara *et al*., 2004).

This ecosystem is of great interest for exploring the response of the bacterial community to a stress produced by crude oil pollution, since the sediments of the retention basin were exposed for several decades to chronic oil pollution. The bacterial community inhabiting the subsurface sediment from the Etang-de-Berre retention basin is particularly well adapted to, and depends on, the oil contamination (Païssé *et al*., 2008; Païssé *et al.*, 2010). As a consequence, the bacterial community inhabiting these sediments presents important capacities to cope with petroleum. Indeed, the dominant *alkB* genes detected were closed to the *alkB* of *Marinobacter aquaeolei* isolated from an oil-producing well (Márquez and Ventosa, 2005; Stan-Lotter, 1999). Interestingly, *alkB* genes related to *Alcanivorax borkumensis*, which has been described as an obligate alkanotroph (Yakimov *et al*., 1998), was detected abundant in the mRNA of the microcosms but was undetectable in the sediments DNA. The bloom of *Alcanivorax* genera in response to oil contamination has been already mentioned (Head *et al*., 2006). Nevertheless, we did not find a clear relationship between the oil contamination and the expression of the *alkB* genes. In our environment, *alkB* is revealed to be a bad indicator of the response to the pollution. *alkB* presence and diversity are frequently used as indicator for investigations concerning the petroleum and the alkane degradation, nevertheless, other enzymes may be involved in the alkane activation such as the cytochrome P450 (Van Beilen *et al*., 2006). This study lead us to conclude that *alkB* gene

presence, diversity and expression are not specific enough or directly relevant for investigating oil contamination or bacterial community adaptation to oil.

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Table 1: Validation of designed primer sets by *alkB* gene amplification from complex environmental matrix. All the primer sets used were previously selected by *alkB* gene amplification from the DNA of *Marinobacter hydrocarbonoclasticus* SP17 strain. The amplicon size referred to the *alkB* gene sequence of *Pseudomonas putida* OCT plasmid (AJ245436). - no amplification; + low amplification; ++ moderate amplification and +++ high amplification.

Primer set used in first round of PCR	Primer set used in second round of PCR	Amplicons size (bp)	Amplification yield
AlkB1F-AlkB1R	AlkB2F-AlkB1R	414	**++**
	AlkB3F-AlkB1R	344	**-**
AlkB2F-AlkB1R	AlkB3F-AlkB1R	344	**+**
AlkB3F-AlkB2R	AlkB3F-AlkB1R	344	**++**
AlkB3F-AlkB3R	AlkB3F-AlkB1R	344	**++**
	AlkB3F-AlkB2R	477	**+++**

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Table 2: Detection by RT-PCR of the *alkB* mRNA in unoiled (B-OIL) and oiled (B+OIL) microcosms. RT-PCR was performed with the AlkB3F-AlkB3R and AlkB3F-AlkB2R primer sets. + and - indicate respectively detection or absence of detection of *alkB* transcripts in slurry sample.

Condition	replicate	0h	1h	2h	3h	6h	12h	24h	2d	3d	4d	5d	7d	9d	12d	14d
B-OIL microcosm	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	C	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
B+OIL microcosm	A	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-
	B	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-
	C	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-

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Figures

Figure 1: Representation of the relative abundance of T-RFs corresponding to *alkB* genes and transcripts detected in *in situ* and in 6H B+OIL samples. The analyses were based on 5'-end *Sau3A1* T-RFLP patterns.

Figure 2: Amino acid (A) and nucleotide (B) positions of designed primers on *alkB* and *alkM* sequences. Others primers developed by Smits *et al*. (1999) and Whyte *et al*. (1996) are also represented on the pattern.

Figure 3: Phylogenetic tree based on the alignment of deduced amino acid sequences (121 amino acids) of amplified *alkB* genes from the Etang-de-Berre sediment and *alkB* transcripts of microcosms. All amplified products were obtained by half-nested PCR (AlkB3F-AlkB3R follow by AlkB3F-Alk2R). The numbers in parenthesis closed to the sequence name indicate the percentage of clones in the corresponding library. The numbers on the right of the tree indicate the percentage of identity between all the amplified sequences in the group delimited by bracket. The scale bar corresponds to 0.05 substitutions per amino acid position. Percentages of 1000 bootstrap re-sampling that supported the branching orders in each analysis are shown above or near the relevant nodes

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Figure 1

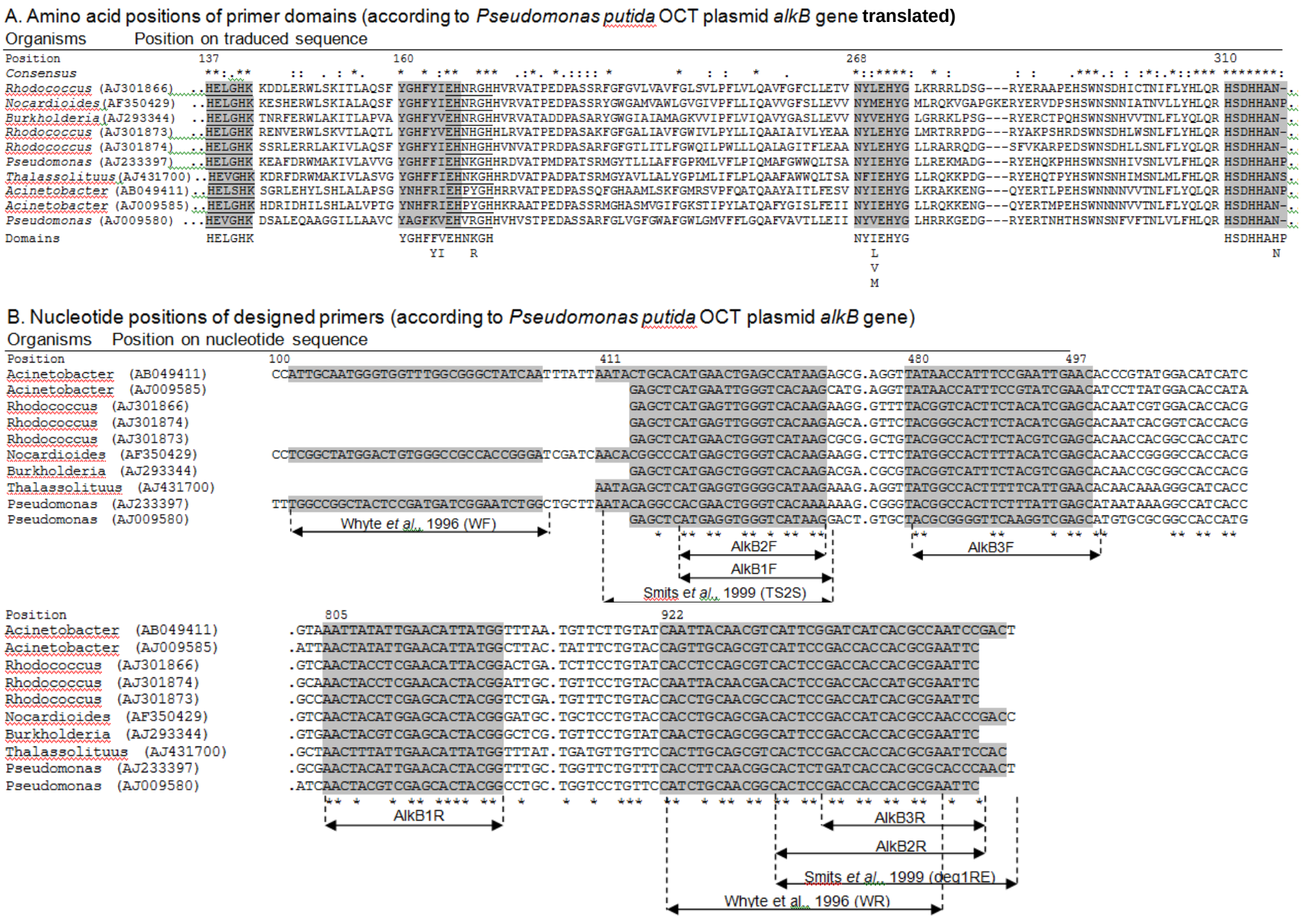

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Figure 2

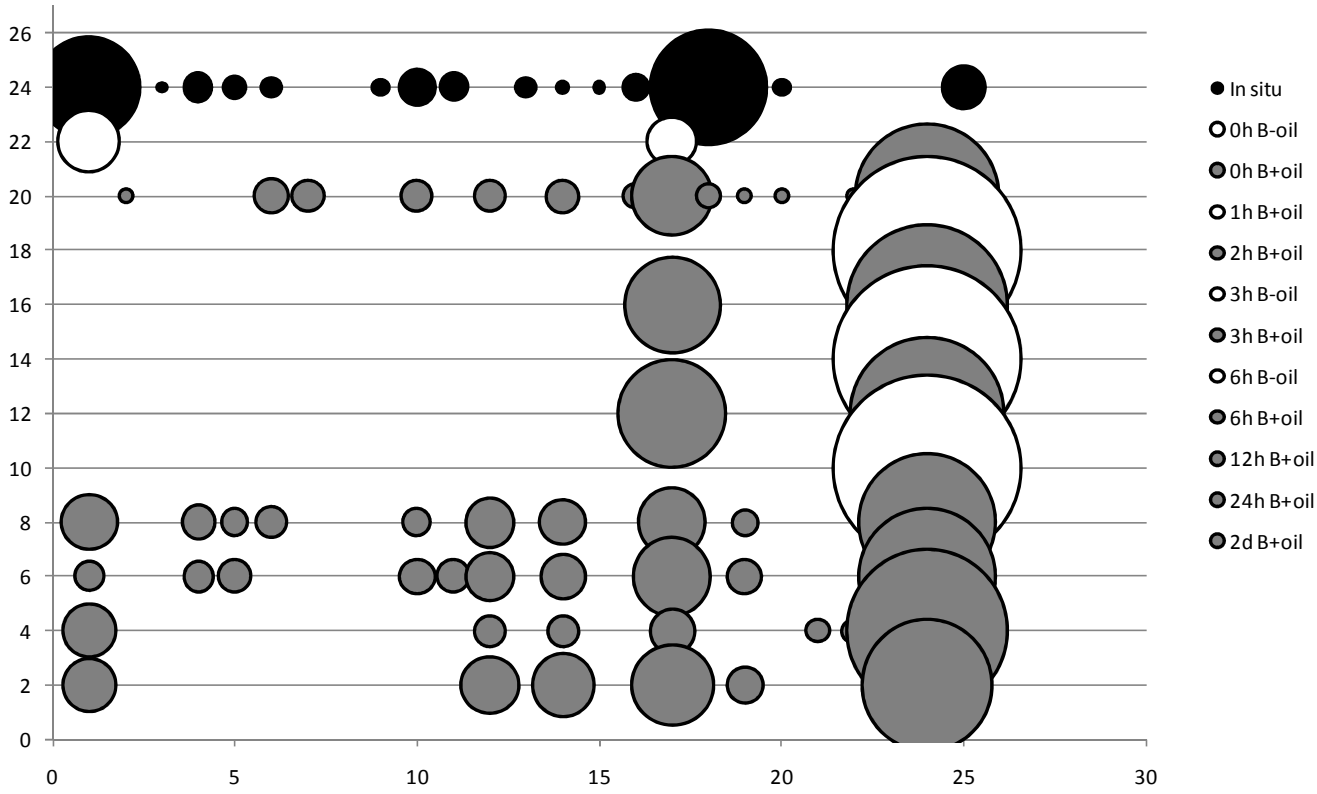
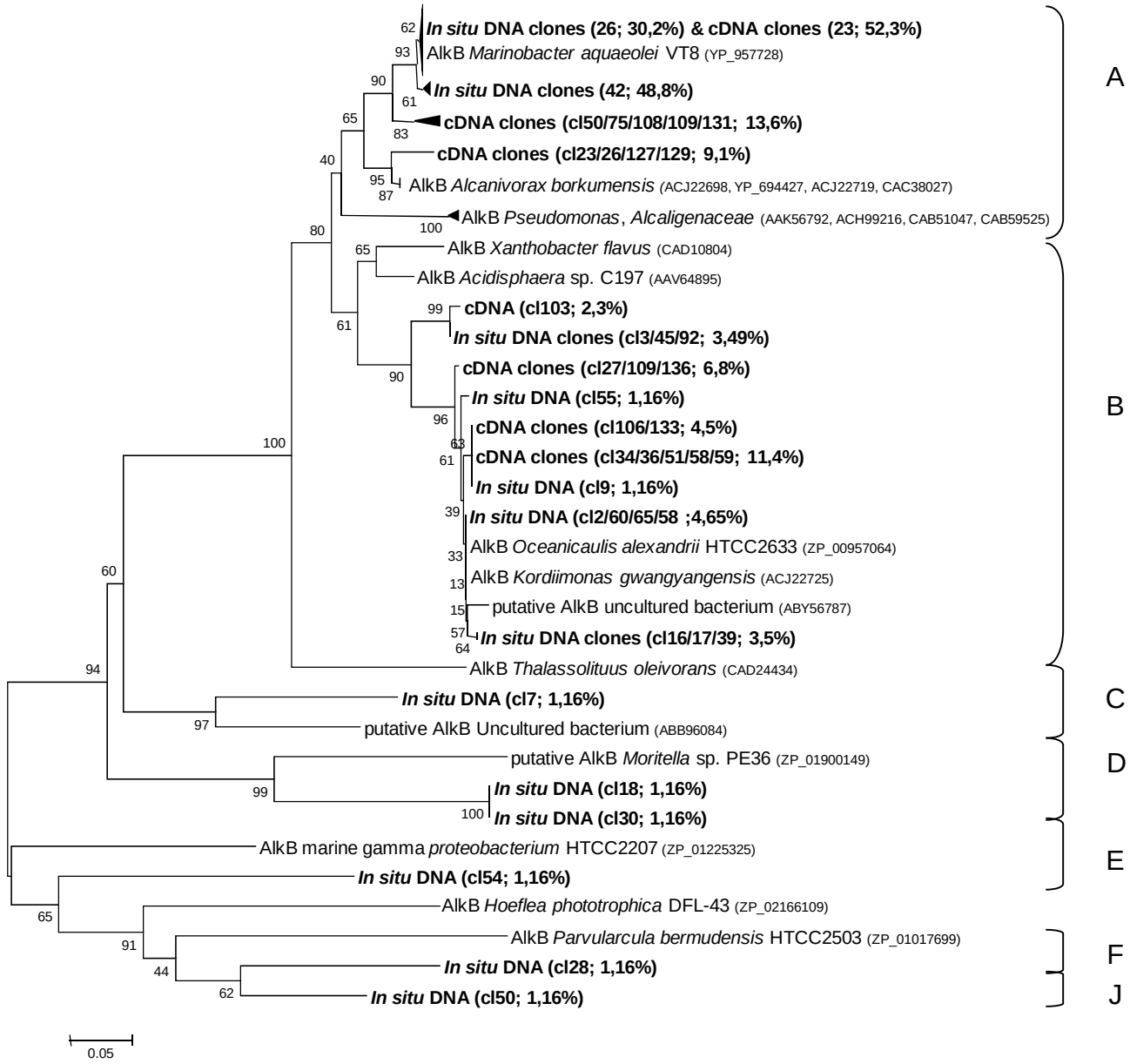


Figure 3


***In situ* DNA clones (26; 30,2%) & cDNA clones (23; 52,3%)**
AlkB *Marinobacter aquaeolei* VT8 (YP_957728)
***In situ* DNA clones (42; 48,8%)**
cDNA clones (cl50/75/108/109/131; 13,6%)
cDNA clones (cl23/26/127/129; 9,1%)
AlkB *Alcanivorax borkumensis* (ACJ22698, YP_694427, ACJ22719, CAC38027)
AlkB *Pseudomonas*, *Alcaligenaceae* (AAK56792, ACH99216, CAB51047, CAB59525)
AlkB *Xanthobacter flavus* (CAD10804)
AlkB *Acidisphaera* sp. C197 (AAV64895)
cDNA (cl103; 2,3%)
***In situ* DNA clones (cl3/45/92; 3,49%)**
cDNA clones (cl27/109/136; 6,8%)
***In situ* DNA (cl55; 1,16%)**
cDNA clones (cl106/133; 4,5%)
cDNA clones (cl34/36/51/58/59; 11,4%)
***In situ* DNA (cl9; 1,16%)**
***In situ* DNA (cl2/60/65/58 ;4,65%)**
AlkB *Oceanicaulis alexandrii* HTCC2633 (ZP_00957064)
AlkB *Kordiimonas gwangyangensis* (ACJ22725)
putative AlkB uncultured bacterium (ABY56787)
***In situ* DNA clones (cl16/17/39; 3,5%)**
AlkB *Thalassolituus oleivorans* (CAD24434)
***In situ* DNA (cl7; 1,16%)**
putative AlkB Uncultured bacterium (ABB96084)
putative AlkB *Moritella* sp. PE36 (ZP_01900149)
***In situ* DNA (cl18; 1,16%)**
***In situ* DNA (cl30; 1,16%)**
AlkB marine gamma *proteobacterium* HTCC2207 (ZP_01225325)
***In situ* DNA (cl54; 1,16%)**
AlkB *Hoeflea phototrophica* DFL-43 (ZP_02166109)
AlkB *Parvularcula bermudensis* HTCC2503 (ZP_01017699)
***In situ* DNA (cl28; 1,16%)**
***In situ* DNA (cl50; 1,16%)**
0.05
A
B
C
D
E
F
J