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Diverse Bacterial Groups Contribute to the Alkane Degradation Potential of Chronically Polluted Subantarctic Coastal Sediments

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Abstract We aimed to gain insight into the alkane degradation potential of microbial communities from chronically polluted sediments of a subantarctic coastal environment using a combination of metagenomic approaches. A total of 6178 sequences annotated as alkane-1-monooxygenases (EC 1.14.15.3) were retrieved from a shotgun metagenomic dataset that included two sites analyzed in triplicate. The majority of the sequences binned with AlkB described in Bacteroidetes (32±13 %) or Proteobacteria (29±7 %), although a large proportion remained unclassified at the phylum level. Operational taxonomic unit (OTU)-based analyses showed small differences in AlkB distribution among samples that could be correlated with alkane concentrations, as well as with site-specific variations in pH and salinity. A number of low-abundance OTUs, mostly affiliated with Actinobacterial sequences, were found to be only present in the most contaminated samples. On the other hand, the molecular screening of a large-insert metagenomic library of intertidal sediments from one of the sampling sites identified two genomic fragments containing novel *alkB* gene sequences, as well as various contiguous genes related to lipid metabolism. Both genomic fragments were affiliated with the phylum Planctomycetes, and one could be further assigned to the genus *Rhodopirellula* due to the presence of a partial sequence of the 23S ribosomal RNA (rRNA) gene. This work highlights the diversity of bacterial groups contributing to the alkane degradation potential and reveals patterns of functional diversity in relation with environmental stressors in a chronically polluted, high-latitude coastal environment. In addition, alkane biodegradation genes are described for the first time in members of Planctomycetes.

Keywords Alkane monooxygenase · Coastal sediments · Bacteroidetes · Actinomycetes · Planctomycetes · Metagenomics

Introduction

Coastal environments in southern Patagonia are being increasingly affected by anthropogenic activities, such as oil extraction and transportation, maritime traffic, port operations, as well as urban runoffs from fast-growing human settlements. These activities have generated localized chronic oil pollution in certain regions along the coast, as it is the case of Ushuaia Bay, Tierra del Fuego Island, Argentina [1,2]. In addition to hydrocarbon pollution, low temperatures, high UV radiation levels [3], as well as other anthropogenic impacts such as nutrient load [4] and other sources of pollution synergistically contribute towards creating a challenging environment for coastal microbial communities, which could ultimately affect hydrocarbon biodegradation processes [5]. Recent studies have shown that coastal sediments of Ushuaia Bay harbor a diverse alkane-degrading bacterial community, highly adapted to these extreme conditions [6]. Furthermore, oil exposure

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experiments analyzed using PCR-based approaches demonstrated that, in addition to archetypical marine obligate hydrocarbon degraders [7], other less known bacterial populations might also play important roles in hydrocarbon natural attenuation in this ecosystem [6].

Alkanes are major crude oil components, and they are also produced by a variety of organisms, including some plants, algae, and microorganisms [8]. They are highly reduced molecules that can serve as carbon and energy sources for microorganisms, although their effective utilization presents certain challenges. As they are relatively inert, these molecules require high activation energy, and their low water solubility affects their bioavailability [9]. Despite these limitations, various microorganisms have the ability to utilize alkanes as substrates, using different uptake strategies followed by their chemical activation via highly specific enzymatic complexes [9,10].

In general, the alkane-activating enzymes oxidize the terminal methyl group of an alkane, generating a primary alcohol, which is then oxidized by subsequent enzymes to produce the corresponding aldehyde and fatty acid that enters the general cell metabolism through β-oxidation [11]. Of the various enzymes known to catalyze the aerobic activation of alkanes, AlkB-type alkane hydroxylases are among the most widely represented in oil-degrading bacteria [11,12]. They are multicomponent enzyme systems composed of an integral-membrane non-heme diiron monooxygenase (alkane-1-monooxygenase (AlkB)), and two soluble electron transfer proteins: rubredoxin (AlkG) and rubredoxin reductase (AlkT) [11]. Other regulatory and transport proteins also take part in the pathway [11]. Alk systems have been extensively studied due to their relevance for aliphatic hydrocarbon biodegradation. For instance, the *alkB* genes have been used as molecular markers to analyze the diversity and abundance of alkane-degrading bacteria in a variety of natural environments, including soils [13,14], coastal and deep-sea sediments [6,15,16], and seawater [17,18]. In addition, *alkB* genes have been used as indicators for the microbial prospection of oil and gas (MPOG [19]), and alkane hydroxylases are considered promising biocatalysts [20]. Recently, an in-depth analysis of genomes and metagenomes showed that the diversity of *alkB* genes is significantly higher than previously estimated using PCR-based approaches [21].

The aim of this work was to further our knowledge of the identity and diversity of microorganisms with the potential to aerobically degrade alkanes in a subantarctic coastal environment, through the metagenomic analysis of the functional biomarker gene *alkB*. In order to obtain a more complete representation of the diversity of *alkB* genes, as well as to gain insight into their genomic context, we used a combination of metagenomic approaches including the shotgun sequencing of sediment DNA and the analysis of a large-insert fosmid library. Large-scale amplicon sequencing of 16S ribosomal RNA (rRNA) genes was used to place this information in the context of the bacterial community structure. This study revealed the important contribution of members of the Bacteroidetes and Proteobacteria phyla in the alkane-degrading community of these subantarctic sediments, showed a correlation of AlkB distribution patterns with alkane concentrations, and allowed us to describe for the first time putative genes related to alkane biodegradation in members of the Planctomycetes phylum.

Materials and Methods

Sample Collection and Chemical Analysis

Subtidal and intertidal sediment samples were retrieved from the coast of Ushuaia Bay (Tierra del Fuego Island, Argentina), next to Ushuaia city. Subtidal sediments (11.3–12.3 m bathymetry, 0–5 cm sediment depth) were collected in the proximity of two piers in December of 2008: (a) Commercial pier (MC, 54° 48.656′ S 68° 17.731′ W, samples ARG01-ARG03) and (b) Orion plant oil jetty (OR, 54° 48.256′ S 68° 17.296′ W, samples ARG04-ARG06). Triplicate sediment samples were manually collected by scuba diving using acrylic cores with an inner diameter of 4.4 cm. Intertidal sediments (0–3 cm) were collected from OR site in June of 2007 (OR07 sample). Sampling was performed using similar acrylic cores along the low tide line at ten random points, which were combined and thoroughly mixed. All samples were kept at 4 °C during transport to the laboratory and were stored at −80 °C for DNA-based analysis or −20 °C for chemical analysis. Aliphatic hydrocarbon content of subtidal sediment samples was determined by gas chromatography equipped with a mass spectrometer (GC/MS), as previously described [22]. Aliphatic biodegradation diagnostic indices [2,6] calculated for these samples were: the ratio between *n*-C17 alkane and pristane (n-C17/Pr) and the ratio between n-C18 alkane and phytane (n-C18/Phy).

16S rRNA Gene Amplicon Sequencing

DNA was extracted from subtidal samples ARG01-ARG06, and the V6-V8 region of the 16S rRNA gene was amplified using primers 926F and 1392R (5′ AAACTYAAAKG AATTGAC 3′/5′ GGACGGGCGGTGTGTRC 3′), as previously described [23]. Sequences were generated by pyrosequencing with Roche 454 GS-FLX platform with Titanium chemistry at the facilities of the United States Department of Energy (DOE) Joint Genome Institute (JGI, http://genome.jgi.doe.gov), following the manufacturer's instructions. Sequence analysis was performed with QIIME v.1.9.1 [24] and OTU-based ecological analyses were performed in R software

environment (www.r-project.org). Details are provided in Online Resource 1.

Analysis of Putative AlkB Sequences Identified in the Shotgun Metagenomic Dataset

Metagenomic DNA sequences were generated from DNA extracted from sediment samples ARG01-06 at the United States Department of Energy (DOE) Joint Genome Institute (JGI, http://genome.jgi.doe.gov). The metagenome sequencing was performed using paired - end sequencing technology with each sample run on one lane of the Illumina HiSeq™ 2000 platform, and annotated by the integrated microbial genomes and metagenomes (IMG/M) annotation pipeline [25]. The six metagenomes were searched for alkane - 1-monooxygenase (EC 1.14.15.3) sequences by gene product name. For taxonomic assignment, standalone blastp was performed against a custom database including all AlkB sequences identified in isolated strains available at the *fungene* database (http://fungene.cme.msu.edu) as to December 1st 2014. The results were further analyzed with MEGAN4 software [26]. Putative AlkB sequences were grouped into OTUs defined at 80 % identity (at the amino acid level) with a closed reference OTU picking method against the fungene database, using CD-HIT-2D [27]. Diversity estimators were calculated in R environment (Online Resource 1). For phylogenetic placement, the metagenomic sequences were placed in a reference Maximum Likelihood tree built from a reference alignment, using Randomized Accelerated Maximum Likelihood and Evolutionary Placement Algorithm (RAxML-EPA [28]). Details of all these methods are provided in Online Resource 1.

Metagenomic Fosmid Library Construction and Analysis

The composite sample OR07 was used for the construction of a metagenomic fosmid library, as previously described [29]. Clones containing *alkB* genes were screened by PCR using a broad specificity primer set (AlkB484F/AlkB824R [30], with cycling conditions as previously described [6]). The identified clones were induced to high copy number, the fosmids were purified using QIAGEN Plasmid Mini Kit (QIAGEN, CA), and fully sequenced using Roche 454 GS-FLX Titanium technology (INDEAR, Rosario, Argentina). Sequences were assembled using Newbler 2.6 software. Gene prediction and annotation was performed using automatic annotation (RAST [31]) and further manually curated. Taxonomic assignment of the metagenomic fragments was performed by similarity-based (MEGAN4 [26]) and composition-based (PhyloPythiaS [32]) methods. For details, see Online Resource 1.

Accession Numbers

The sequences of the genomic fragments from the fosmids were deposited in Genbank under accession numbers KP216408 (fosmid No. 401) and KP216409 (fosmid No. 964). The 16S rRNA gene reads obtained by amplicon pyrosequencing were deposited in SRA under accession numbers SRR1974485 (ARG01), SRR1976076 (ARG02), SRR1976077 (ARG03), SRR1976079 (ARG04), SRR1976082 (ARG05), and SRR1976083 (ARG06). The metagenomes analyzed in this work correspond to IMG genome IDs 3300000122, 3300000242, 3300000118, 3300000121, 3300000131 and 3300000125.

Results

Relation Between Alkane Pollution and Community Structure in Ushuaia Bay Sediments

Aliphatic Hydrocarbon Content

Triplicate sediment samples were obtained at two sites in Ushuaia Bay (MC and OR) distanced approximately 500 m. All samples showed moderate levels of aliphatic hydrocarbons, although values were higher and more variable in sediments of OR site, ranging from 0.27 to 5 μg/g of wet sediment (Table 1). In accordance with higher pollution levels, dissolved oxygen concentrations on the top layer of the sediment were lower at this site (Table 1). Biodegradation diagnostic indices showed low values, which suggests ongoing biodegradation processes (Table 1).

Bacterial Community Structure

After preprocessing, a total of 57,966 high-quality 16S rRNA gene sequences (~400 bp, 3532 to 12,485 sequences per sample) were obtained from amplicon sequencing of metagenomic DNA from Ushuaia Bay sediments. The dataset was subsampled to 3500 sequences per sample in order to allow the comparison of ecological estimators at the same sequencing effort. At this threshold, coverage values were 94–95 % for all samples (Online Resource 2). Ecological estimators calculated for these communities showed no significant differences by site (Student's *t* test *p* values=0.5 for both Chao1 and Shannon's indexes). The most abundant bacterial groups present in Ushuaia Bay sediments were the Proteobacteria (mainly belonging to Delta- and Gammaproteobacteria classes), followed by Bacteroidetes (mainly Flavobacteria) Verrucomicrobia and Firmicutes (Fig. 1a). Dominant taxa were shared among samples: unclassified Flavobacteriaceae (22.7±1.5 %), unclassified Desulfurobulbaceae (Deltaproteobacteria, Desulfurobacterales, 23.5±1.4 %), unclassified Desulfuromonadaceae

Table 1 Alkane concentration and other physico-chemical variables of Ushuaia Bay sediment samples

Site	Sample	Depth (m)	Temp (°C)	Sal (‰)	pH	DO (mg/l)	Total Ali (μg/g ws)	*n*-C17/Pr	*n*-C18/Phy
MC	ARG01	11.3	8.52±0.04	29.42±0.04	8.00±0.00	14.88±0.06	0.09	0.68	1.73
	ARG02	11.3	8.66±0.05	29.34±0.05	8.01±0.01	14.96±0.18	0.09	2.72	0.63
	ARG03	11.3	8.50±0.00	29.42±0.04	7.99±0.00	14.29±0.09	0.08	1.03	1.01
OR	ARG04	12.3	8.74±0.05	29.24±0.05	7.68±0.05	13.89±0.30	0.27	1.02	0.83
	ARG05	12.3	8.60±0.00	29.30±0.00	7.79±0.01	13.26±0.05	0.57	0.92	0.78
	ARG06	12.3	8.60±0.00	29.30±0.00	7.84±0.01	13.23±0.04	5.01	1.54	1.65
	OR07	0	nd	nd	nd	nd	8.74[a]	1.27[a]	1.34[a]

Temp temperature, *sal* salinity, *DO* dissolved oxygen, *Total Ali* total aliphatic hydrocarbon concentrations (defined as the sum of *n*-alkanes, branched and cyclic alkanes), *ws* wet sediment, *n-C17/Pr* ratio between *n*-C17 alkane and pristane, *n-C18/Phy* ratio between *n*-C18 alkane and phytane, *nd* not determined

[a] Previously reported [6]

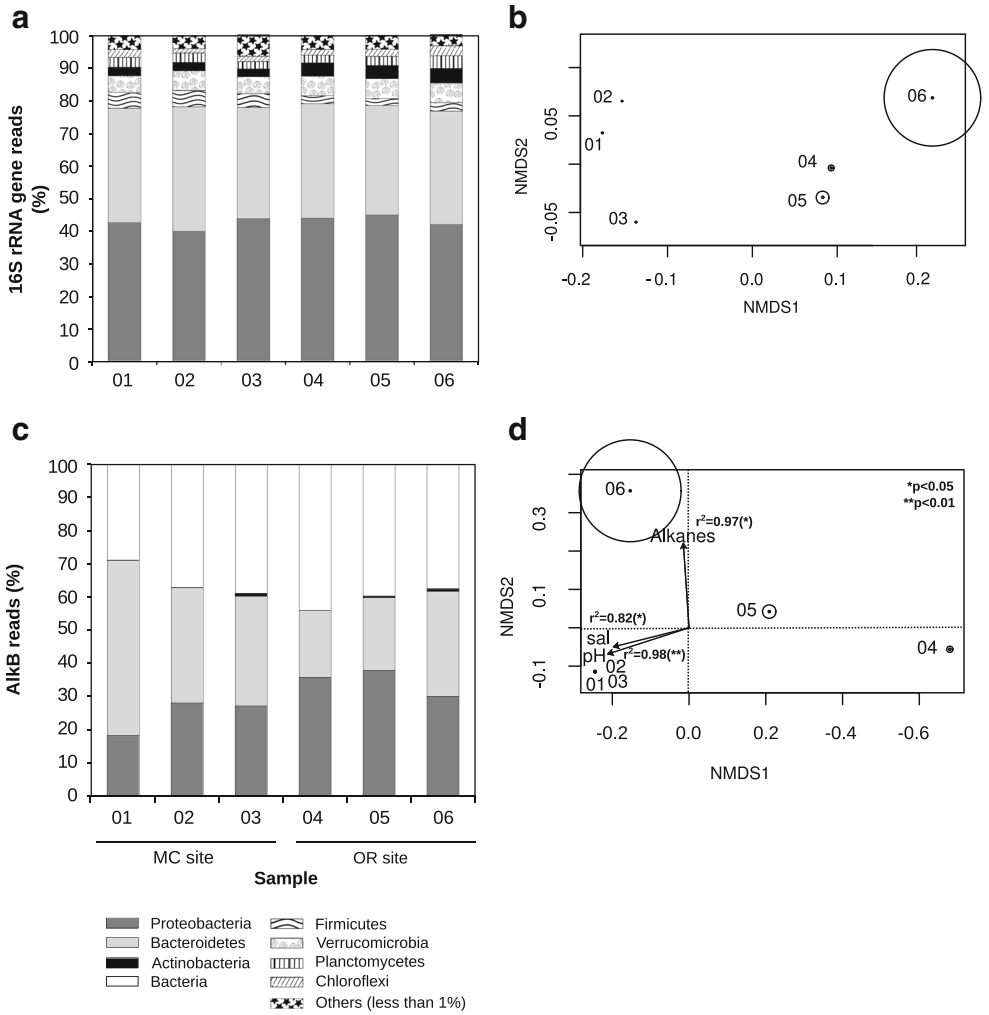

Fig. 1 Phylogenetic and functional gene-based analysis of bacterial communities from Ushuaia Bay sediments. **a** Distribution of major bacterial phyla, based on the classification of rRNA gene amplicons. *Others*: bacterial groups accounting for less than 1 % of the reads. Samples from MC site: 01–03; samples from OR site: 04–06. **b** Ordination (non-metric multidimensional scaling) of bacterial communities based on OTUs defined at 97 % identity threshold. Circle size is proportional to the aliphatic hydrocarbon content of the sample. Stress <0.01 **c** Classification of AlkB sequences identified in the shotgun sequence dataset. Analysis were performed with MEGAN software [26]. *Bacteria*: AlkB sequences that could only be assigned as belonging to domain Bacteria. **d** Ordination of communities based on AlkB OTUs defined at 80 % identity at the amino acid level. Environmental vectors resulting in significant correlations with major ordination axes are superimposed in the plot. Correlation coefficients are shown with the corresponding significance values. Stress <0.01. Analyses were performed in R package *vegan*

(Deltaproteobacteria, Desulfuromonadales, 2.9±0.4 %), unclassified Verrucomicrobia group WCHB1-41 (2.3±0.5 %), *Psychromonas* (Gammaproteobacteria, Alteromonadales, Psychromonadaceae, 1.6±0.3 %), among others (Online Resource 3). No significant differences in the relative abundances of taxa at family or genus levels were found between sites (Mann–Whitney U test, Bonferroni-corrected p value=1). At the OTU level, Bray-Curtis similarities ranged from 0.57 (between ARG03-ARG05) to 0.65 (ARG04-ARG05). Ordination based on OTUs showed no correlation between bacterial community structure and alkane concentration, or with any other of the measured environmental variables (Fig. 1b).

Putative AlkB sequences identified in the shotgun metagenomic dataset

A total of 6178 coding sequences, annotated as alkane-1-monooxygenase (EC 1.14.15.3) and ranging from 23 to 350 amino acids (median=45), were retrieved from the shotgun metagenomic dataset of the six subtidal sediment samples from OR and MC sites. The relative abundance of the putative AlkB sequences per metagenome ranged from 1.4 to 7.2 sequences per 10^6 reads (corrected by scaffold read depth in the case of assembled reads). No correlation was found between the relative abundance of the metagenomic sequences and aliphatic hydrocarbon concentrations. However, a weak correlation was observed between the absolute number of putative AlkB sequences and the size of the metagenome (total number of reads), suggesting an undersampling effect (data not shown). Most sequences were either assigned by MEGAN to Proteobacteria (29±7 %), or to the Bacteroidetes-Chlorobi group (32±13 %), while 38±5 % of the sequences could only be classified as related to Bacteria (Fig. 1c).

The metagenomic sequences were further clustered into operational taxonomic units (OTUs) with a distance threshold of 0.2 (80 % sequence identity at the amino acid level). In order to minimize OTU overestimation due to poor sequence overlapping, we selected to use a closed OTU assignment against a curated AlkB database (*fungene*, see "Supplementary Materials and Methods" Online Resource 4). Only 1330 of the 6178 sequences clustered with the reference sequences at the selected threshold. Therefore, the number of identified OTUs (140) should be considered a highly conservative estimation of the number of sequence variants present in this metagenomic dataset. Dominant OTUs were related to sequences identified in members of the Flavobacteria and Gammaproteobacteria, e.g., AlkB from Flavobacteriales bacterium ALC-1 (OTU0, 19±7 % of the sequences), marine Gammaproteobacterium HTCC2207 (OTU1, 19±6 %), *Polaribacter* sp. MED152 (OTU 8±4 %), and *Maribacter* sp. HTCC2170 (OTU3, 6±4 %, Online Resource 5). Overall, these OTUs accounted for 55 % of the metagenomic sequences assigned into OTUs. No significant differences were found between the two sampling sites with regard to the estimated number of OTUs (Chao's index, 98±11 and 103±5 for MC and OR, respectively; Student's t test p value=0.3). However, small differences were found in evenness, with OTUs more evenly distributed in OR samples than in MC samples (Pielou's evenness, 0.72±0.01 and 0.82±0.05 for MC and OR, respectively; Student's t test p value=0.08). Bray-Curtis similarity values among these samples ranged from 0.24 (ARG04-ARG06) to 0.54 (ARG02-ARG03). In contrast to the results obtained with OTUs of bacterial 16S rRNA genes, an ordination of samples based on AlkB OTUs resulted in significant correlations with environmental factors, including pH, salinity, and alkane concentrations (Fig. 1d). Fifteen distinctive OTUs were found to be only present in the most contaminated samples (i.e. only in ARG06 or in ARG05 and ARG06). These OTUs were related to sequences from Actinobacteria (10 OTUs, similar to AlkB from *Rhodococcus* [OTU100, OTU102, OTU117], *Thermomonospora* [OTU109], *Mycobacterium* [OTU55, OTU112, OTU93], *Nocardioides* [OTU64], and *Amycolicicoccus* [OTU92, OTU99]), Alphaproteobacteria (3 OTUs, similar to AlkB from *Roseobacter* [OTU72], *Oceanicaulis* [OTU107], and *Phaeobacter* [OTU116]), and Gammaproteobacteria (2 OTUs, affiliated with *Alcanivorax* [OTU73] and *Psychrobacter* [OTU88]). Although distinctive of the most polluted samples, these OTUs were detected at relatively low abundances (0.47 to 1.29 %, Online Resource 5).

A subset of 116 sequences longer than 80 amino acids in length and containing at least one of the conserved histidine-containing motifs described for this enzyme family [33] were further analyzed phylogenetically. As the lack of overlapping among the metagenomic sequences precluded an overall alignment, they were independently positioned in a fixed AlkB reference tree containing representative sequences of all clusters defined for AlkB [21], by using the Evolutionary Placement Algorithm [28]. The results are shown in Fig. 2 (the complete names of the reference sequences are detailed in Online Resource 6). Cluster V (mainly putative AlkB sequences from genomes of Bacteroidetes) contained the majority of the metagenomic sequences, followed by cluster IV (a diverse cluster containing sequences similar to archetypical AlkB1 from *Alcanivorax borkumensis* and *Pseudomonas putida* GPo1) and cluster VII (recently described, with putative sequences obtained from the genomes of members of the Rhodobacterales, Alphaproteobacteria [21]). The analysis of the full dataset showed a similar distribution pattern of sequences across clusters (Online Resource 7). Moreover, the higher coverage allowed the detection of low abundance sequences distributed all along the phylogenetic tree, such as the ones placed in Cluster I from Actinobacteria.

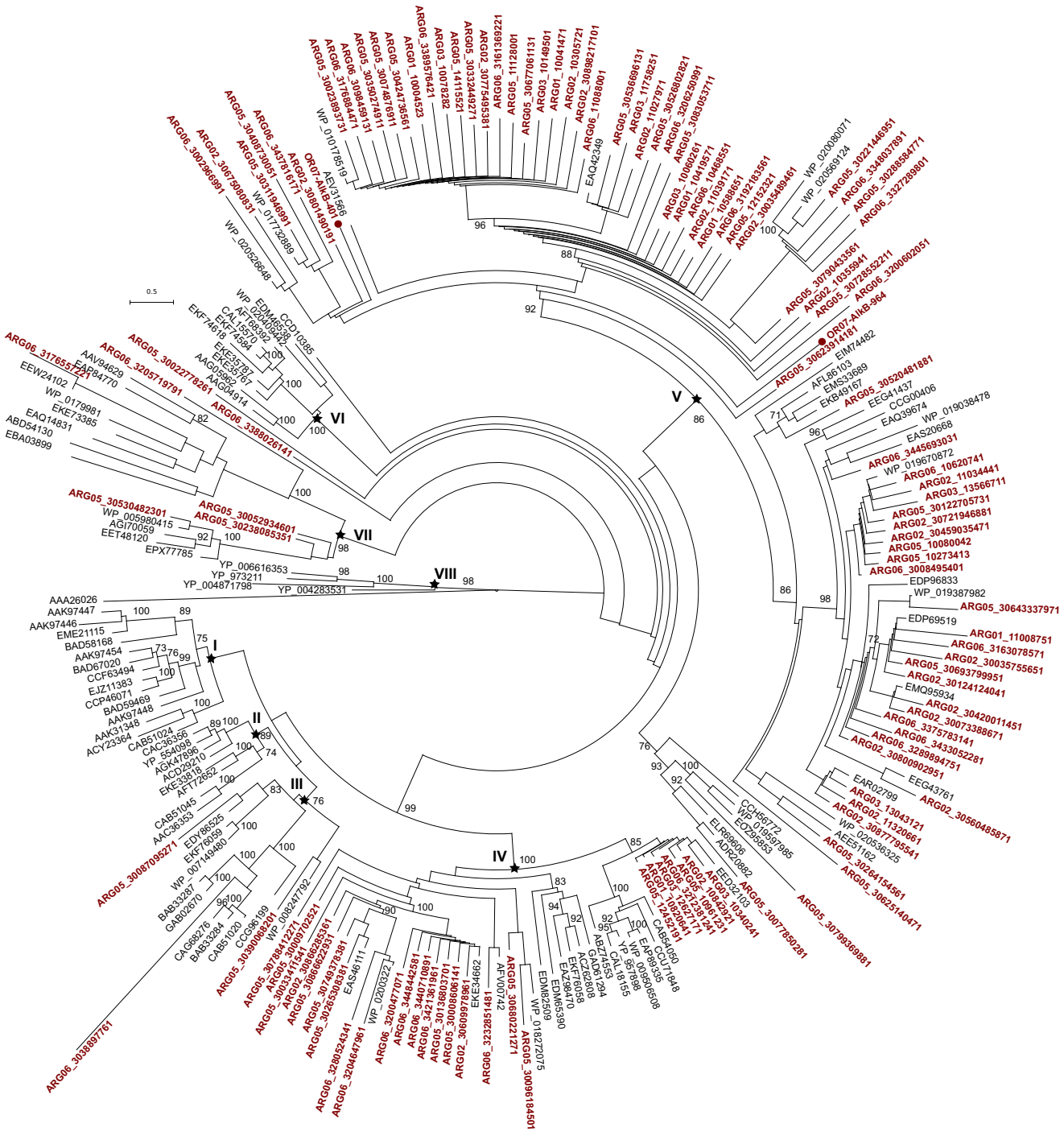

Fig. 2 Phylogenetic placement of putative AlkB sequences identified in the metagenome of Ushuaia Bay sediments. The tree was constructed by Maximum Likelihood in RAxML v.8.2.3. Reference sequences are indicated with the GenBank accession number, and metagenomic sequences are indicated with their gene ID preceded by sample name (ARG01-06). Complete names of reference sequences are available in Online Resource 6. AlkB sequences from this study are shown preceded by sample name (ARG01-06, OR07). The clusters defined by Nie and collaborators [21] are shown in *roman numerals*. The *likelihood weight ratio* (a measure of the certainty of the placement of the sequences [69]) was 0.66±0.26. Only bootstrap values ≥70 % (100 repetitions) placed in the best maximum likelihood tree are shown above the corresponding branches. Xylene monooxygenase catalytic subunit (XylM) from *Pseudomonas putida* TOL plasmid (AAA26026) was used as outgroup. The *scale bar* represents 0.5 estimated amino acid substitutions per site

Analysis of Metagenomic Fragments Carrying Putative *alkB* Genes Identified in a Fosmid Library of Ushuaia Bay Sediments

Analysis of AlkB Sequences

A large-insert metagenomic library was constructed from intertidal sediment sample OR07 and screened by PCR using a broad specificity primer set targeting *alkB* genes [6,30]. Two fosmid clones (No. 401 and 964) were identified using this approach. The corresponding putative AlkB sequences, AlkB-401 and AlkB-964, presented the four conserved histidine-containing motifs, as well as six predicted transmembrane segments, as described for alkane monooxygenases [33,34] (Online Resource 8). These two sequences shared 47 % identity and 73 % similarity at the amino acid level. Their closest matches in blastp searches against the NCBI database corresponded to putative AlkB sequences predicted in members of Bacteroidetes phylum, such as *Flexithrix dorotheae* (WP_020526648, 55 % identity and 75 % similarity with AlkB-401) and *Nafulsella turpanensis* (WP_017732889, 51 % identity and 70 % similarity with AlkB-964). They were placed within Cluster V of the AlkB phylogenetic tree, although they did not clearly cluster with any of the reference sequences (Fig. 2).

Analysis of the Genomic Context of the Putative alkB Genes

The use of a fosmid vector for the construction of the metagenomic library allowed the identification of longer metagenomic fragments carrying putative *alkB* genes, in comparison with the shotgun sequencing strategy (Table 2). Gene prediction and annotation of these metagenomic fragments detected 24 and 28 coding sequences (CDS) in fragments No. 401 and 964, respectively. No function could be assigned to 17 of these CDS (Table 2). Overall, 80 % of functionally assigned CDS could be included into COG categories (Online Resource 9). No clusters of *alk* genes were found in these fragments, although genes that could possibly be associated with an alkane degradation pathway were observed in the vicinity of the *alkB* genes. Fragment No. 401 included a putative transcriptional regulator of the TetR family (CDS No. 401-10) and a putative acyl coenzyme A synthetase (CDS No. 401-8) (Fig. 3). The *alkB* gene and the putative regulator were predicted to form an operon, suggesting that this gene could be subjected to regulation. Two other genes that could be related to lipid metabolism were found in the neighborhood of the *alkB* gene: CDS No. 401-5 (putative carboxylic ester hydrolase) and CDS No. 401-7 (putative lipase) (Fig. 3). In fragment No. 964, the *alkB* gene was predicted to form an operon with three other genes: a putative aldehyde dehydrogenase (CDS No. 964-2), a putative phosphotransferase (CDS No. 964-1), and a hypothetical protein (CDS No. 964-3) (Fig. 3, Online Resource 9).

Possible Phylogenetic Origin of the Metagenomic Fragments

The two fragments identified in the fosmid library were found to be affiliated to microorganisms belonging to the Planctomycetes phylum, both by similarity-based (MEGAN, blastn) and composition-based (PhylopythiaS) methods. Both fragments could be classified as belonging to the Planctomycetaceae family, and fragment No. 401 was further identified as belonging to the genus *Rhodopirellula*. A 976 bp sequence corresponding to the 23S rRNA gene present in fragment No. 401 confirmed these results (Online Resource 10). Genes linked to transcription, translation, and related processes (COG categories J, and K) of fragment No. 964 were binned by MEGAN into *Planctomyces maris* (Online Resource 9). These types of genes, also defined as "informational genes," are subject to few or no horizontal gene transfers, and therefore they are considered more reliable than other gene categories for inferring phylogenetic history [35,36].

Members of Planctomycetes form a monophyletic, highly supported cluster in 16S and 23S rRNA-based phylogenies [37,38]. However, the AlkB sequences identified in the fosmids, although divergent, were placed in Cluster V (mainly containing putative AlkB from genomes of members of the Bacteroidetes, Fig. 2). This raises the question about the possibility of horizontal gene transfer as the origin of *alkB* genes in these microorganisms. The presence of a gene potentially encoding a transposase in one of the fosmids (Fig. 3) was an evidence in favor of this hypothesis. However, the average GC content of the fosmid sequences (54–55 %, Table 2) was similar to the average determined for Planctomycetes (55.64 %, as estimated from Planctomycetes genomes available at www.img.jgi.doe.gov), and no anomalies in GC content could be found in the vicinity of the *alkB* gene (data not shown), suggesting that this event, if occurred, was not recent [39]. Interestingly, 22 and 4 AlkB sequences identified in the shotgun metagenomic dataset shared more than 80 % identity at the amino acid level with AlkB from clone No. 401 and No. 964, respectively. These results suggest that uncultured Planctomycetes with alkane-degrading potential might also be members of the microbial community of the subtidal sediments of Ushuaia Bay.

Synteny with Related Genomes

Based on the results of the taxonomic assignment of the metagenome fragments, a comparison was performed with the genomes of the most related microorganisms available at the SEED database. Fosmid No. 401 was compared with the chromosome of *Rhodopirellula baltica* SH 1

Table 2 Metagenome fragments carrying the full-length putative AlkB sequences identified in this study

Source	Name	Size (bp)	GC (%)	Coding sequences	Assigned genes[a]	Blastn[b]	Query overage (%)	Identity (%)
Shotgun metagenomic dataset	TDF_MC_ARG01_113mDRAFT_c1000452	4043	35	3	3	NC_020830 *Polaribacter* sp. MED152	33	68–70
	TDF_MC_ARG01_113mDRAFT_c1004147	1237	33	2	1	NC_020830 *Polaribacter* sp. MED152	52	68
	TDF_OR_ARG03_113mDRAFT_c1007828	1388	34	2	1	NC_020830 *Polaribacter* sp. MED152	51	68
	TDF_OR_ARG05_123mDRAFT_1008004	2824	36	3	2	NC_014472.1 *Maribacter* sp. HTCC2170	70	65–76
Metagenomic library	No. 401	33,897	54	24	15[c]	BX119912 *Rhodopirellula baltica* SH 1	33	64–92
	No. 964	38,325	55	28	20	NC_014962 *Isosphaera pallida* ATCC 43644	12	64–74

[a] Genes where a potential function could be assigned

[b] First match (based on query coverage) obtained when performing blastn search of the complete fragment against the NCBI Genomes database

[c] Included two genes encoding tRNAs and a 23S rRNA gene

(BX119912), and fosmid No. 964 was compared with the genome of *P. maris* DSM 8797 (NZ_ABCE00000000). In both cases, regions with conserved gene order were evident, which were not contiguous in the genomes (Fig. 3). For both metagenome fragments, the regions including the *alkB* genes and their flanking sequences were absent in the related Planctomycetes chromosomes, in accordance with the absence of these genes in the databases.

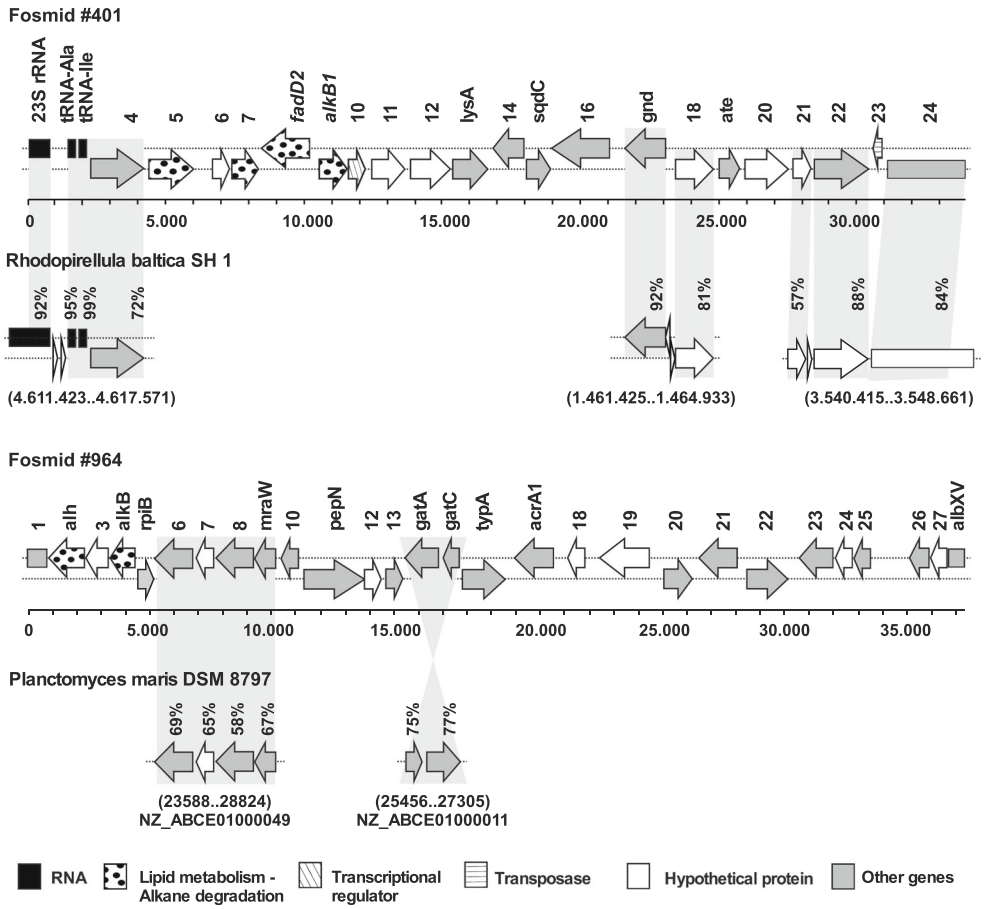

Fig. 3 Gene organization of metagenomic fragments carrying full-length putative *alkB* genes, and comparison with chromosome fragments of related microorganisms. The length and direction of the arrows indicate the relative size and the transcriptional orientation of each gene. When available, gene names are shown above each *arrow*. Otherwise, the identified coding sequences are named with consecutive numbers (for more information, see Online Resource 9). Regions with conserved gene order with respect to the closest genomes are connected in gray. Percent identity at the amino acid level between the compared deduced sequences (or at the nucleotide level for RNA coding genes) is also shown. The relative positions in the closest genomes are shown *between parentheses*

Discussion

In this work, we used two metagenomic approaches to gain insight into the diversity of bacterial populations with alkane-degrading potential from sediments of a subantarctic, chronically polluted coastal environment (Ushuaia Bay, Tierra del Fuego, Argentina [2,5]). In accordance with our previous work at this site [6], these sediments showed moderate levels of petrogenic pollution and ongoing biodegradation processes, as evidenced by the hydrocarbon profiles of the samples. The shotgun sequencing approach allowed us to uncover a higher diversity of putative AlkB sequences than in previous PCR studies performed in similar environments (cold coastal or marine regions) and in the same matrix (sediments). While in this work we detected more than 100 OTUs using a conservative method (closed OTU assignment against reference sequences), previous works analyzing cold sediments identified only 15 to 50 OTUs (defined at the same identity threshold) [15,16], including our previous PCR-based study in Ushuaia Bay sediments [6]. The putative AlkB sequences described here belonged to diverse phyla, including Proteobacteria, Bacteroidetes, and Actinobacteria. In addition, a large-insert metagenomic library allowed us to describe for the first time putative alkane monooxygenase genes in members of the Planctomycetes phylum.

The two sampling sites, although separated at a very short geographic distance, showed differences in pollution levels. This was probably the result of the type of anthropogenic impact that these locations receive, as OR site is located next to a jetty used for loading and unloading refined petroleum products [5], while MC site is near a heavily used commercial pier. There was no correlation of community structure (based on 16S rRNA gene amplicon sequencing) with environmental factors. In contrast, functional community structure (based on putative AlkB sequences) showed subtle patterns that could only be evidenced at a finer level (OTUs), suggesting that pollution has a role in the structuring of the alkane-degrading guild. The distribution of AlkB OTUs was correlated with salinity and pH, as well as with alkane levels. Differences in salinity and pH were probably related with the different coastal impacts of the two sites, such as the influence of different water runoffs [4]. AlkB OTUs were more evenly distributed in the most polluted site, a result which might be explained by the establishment of a more diverse alkane-degrading bacterial community due to its exposure to higher inputs of refined petroleum products. Various low-abundance OTUs were distinctive of the most contaminated samples, most of them affiliated with AlkB sequences from Actinomycetes (*Rhodococcus*, *Thermomonospora*, *Mycobacterium*, *Nocardioides*, and *Amycolicicoccus*). Members of this bacterial group display remarkable alkane degradation capacity, in particular for the most recalcitrant compounds, which are targeted via multiple alkane hydroxylases [9, 21, 40]. They have been associated with natural and enhanced biodegradation of hydrocarbons in coastal sediments [40, 41].

Recent studies have shown that acute pollution events, such as the Deepwater Horizon spill, have clear effects on microbial communities [42]. These are evidenced by dramatic shifts in community structure and functions, such as hydrocarbon degradation and nitrogen cycling pathways. In contrast, in chronically polluted environments the effects of hydrocarbon pollution are not so evident [43]. The analysis of the changes of the alkane-degrading guilds with respect to oil pollution has shown contrasting results in different studies, at least as estimated by the functional biomarker AlkB. In Antarctic sediments, AlkB diversity was found to be higher and its composition different in a pristine site than in a nearby impacted station [15]. In marine natural seeps, on the other hand, *alkB* gene diversity did not vary significantly with alkane levels, although gene copy numbers were observed to be higher near the seeps [16]. In the case of acute pollution events (either natural or experimental), the effects were often more evident, leading to the enrichment of specific *alkB* gene variants [6, 44, 45], or shifts in gene expression as evidenced by a metatranscriptomic approach [22]. In this work, the observed slight differences are probably due to the fact that, although different in sources and load, both analyzed sites are exposed to hydrocarbon inputs [2].

The phylogenetic distribution of putative AlkB sequences suggests an important contribution of diverse taxonomic groups to the alkane-degrading potential of Ushuaia Bay sediments. Although taxonomic binning of functional genes may suffer biases due to the common occurrence of horizontal gene transfer events and the incompleteness of the available databases, this method is still able to provide valuable information, which could be impossible to obtain from a phylogenetic marker gene [21]. However, it must be noted that all the AlkB sequences identified in this study are putative, as they are only based on gene prediction. Among the high diversity of AlkB sequences identified in Ushuaia Bay sediments, a third could be related to putative alkane monooxygenases from genomes from members of the Bacteroidetes. These sequences were as abundant and diverse as Proteobacteria-like AlkB sequences. The phylogenetic distribution of AlkB genes was in accordance with the results obtained by 16S rRNA gene amplicon sequencing, where these groups were also found to be present at high abundances. Overall, these results suggest that members of both Bacteroidetes and Proteobacteria could be important players in the natural attenuation of aliphatic hydrocarbons in this environment by AlkB-mediated alkane activation. Other potential mechanisms could be the aerobic degradation *via* enzymes other than AlkB, as well as anaerobic biodegradation coupled to sulfate reduction by Deltaproteobacteria [46], which were also found to be abundant in the bacterial communities of these sediments.

There is scarce information regarding the role of Bacteroidetes in alkane degradation in marine environments. Members of this bacterial group, especially the Flavobacteria class, are abundant and globally distributed in a variety of marine environments, including seawater and sediments, both coastal and offshore [47]. They have been associated with marine detritus, probably utilizing high molecular weight compounds as carbon and energy sources [48]. Some marine isolates belonging to Flavobacteria are able to utilize long-chain alkanes. One example is the pressure- and solvent-tolerant *Flavobacterium* sp. DS-71, which was isolated from deep-sea sediments and utilizes alkanes up to C_{25} [49]. In a previous study, we observed an increase of various members of the Flavobacteriales when sediments of Ushuaia Bay were exposed to crude oil and amended with nutrients [6]. Moreover, sequences similar to AlkB from members of the Bacteroidetes phylum were identified in that study, although they were much less represented than Proteobacterial-like sequences, probably due to a bias associated with the primers used to amplify the gene fragments [6]. Recently, AlkB sequences from Bacteroidetes were reported to be abundant in a number of metagenomes [21]. More than 60 samples from various environments were analyzed in that study, although only two of the metagenomes were from sediment samples, and only one of them from cold regions (offshore sediments from the Arctic Ocean, Alaska). Therefore, this study represents the first metagenomic analysis of AlkB diversity in coastal subantarctic environments.

We performed a detailed phylogenetic analysis of a subset of the AlkB sequences, in order to place them into the updated classification proposed by Nie and collaborators [21] for this enzyme family. These authors defined eight major AlkB clades (Clusters I to VIII) based on phylogenetic analyses of the sequences identified in available genomes. The AlkB sequences identified were assigned to AlkB cluster IV, cluster VII, and to various groups within cluster V. Among the alkane hydroxylases from cluster IV, the range of substrates has been only studied in *P. putida* GPo1 and *A. borkumensis* SK2 (alkanes from C_5 to C_{12} or C_{13} [34,50]), and in *Alcanivorax dieselolei* B-5 (alkanes up to C_{16} [51]). Sequences from clusters V and VII were only reported in genome projects, so they constitute putative alkane hydroxylases. Interestingly, the hosts of sequences from cluster VII belong mainly to the Roseobacter clade (Alphaproteobacteria, Rhodobacterales). These microorganisms are ubiquitous in the marine environment, and have been related to hydrocarbon biodegradation in a number of studies [52,53], including enrichments with crude oil [54,55], gasoil [56], and individual alkanes [57]. However, to our knowledge, no functional studies of alkane hydroxylases belonging to this cluster have been performed up to date.

The use of a complementary metagenomic approach, the cloning of large DNA fragments in a fosmid vector, allowed us to recover full-length sequences and to analyze their genomic context. We identified two metagenomic fragments, each containing an AlkB sequence, in a fosmid library constructed from sediments of Ushuaia Bay. The taxonomic binning of these fragments suggested that they belong to members of the Planctomycetes phylum. The presence of a partial sequence of a 23S rRNA gene [38] allowed us to unequivocally place the genomic fragment from one of the clones among the members of the aerobic marine genus *Rhodopirellula* [58]. Members of Planctomycetes are widely distributed in marine habitats, where they are thought to utilize complex sulfatated heteropolysaccharides while attached to marine detritus (marine snow), playing an important role in carbon turnover [58,59]. They have also been found to be abundant in marine sediments [60] and associated with algae blooms [61]. This group is also considered to hold features with promising biotechnological applications [37]. Various putative genes probably related to carbohydrate metabolism were present in the metagenomic fragments, suggesting that these uncultured Planctomycetes bear the capability of carbohydrate assimilation. To our knowledge, there is only one report of members of the Planctomycetes phylum with the ability to utilize hydrocarbon compounds as a source of carbon and energy [62]. A putative cytochrome 450 of the CYP153 family, another type of alkane hydroxylase, was recently identified in genomes from *Planctomyces* [21], which is in agreement with the possibility that these microorganisms could be capable of alkane degradation.

Although genes potentially related to the *alk* pathway could be identified next to the putative *alkB* in the metagenomic fragments identified in this study, they did not contain a complete pathway for alkane degradation. Also, no sequences coding for rubredoxins or rubredoxin reductases were found in the fragments. The organization of *alk* genes varies significantly among different alkane-degrading strains. While some specialized genera such as *Alcanivorax* typically harbor clusters of genes encoding all the enzymes that participate in the degradation pathway [63], other alkane-degrading microorganisms have the genes dispersed in the chromosome [64,65]. Also, there was a certain evidence that these genes could be subject to transcriptional regulation, as a putative regulatory protein of the TetR family was identified immediately downstream of the *alkB* gene from fosmid No. 401. This type of transcriptional regulator (sometimes named *alkU*) has been observed next to *alkB* in alkane-degrading bacteria belonging to the Actinobacteria phylum [66], as well as in the marine gammaproteobacterium *Alcanivorax hongdengensis* [67]. In the actinobacterium *Dietzia cinnamea*, the analysis of gene expression suggested that a putative TetR family transcriptional regulator could be responsible for the induction of the alkane hydroxylase (*alkB-rub*) and lipid transporter genes during the late exponential phase [68].

In summary, the combination of metagenomic approaches and deep sequencing of 16S rRNA gene amplicons used in

this study enabled us to gain insight into the phylogenetic and functional diversity of microbial communities from sediments of a subantarctic environment exposed to chronic hydrocarbon pollution. The shotgun sequencing approach allowed the identification of ubiquitous putative AlkB sequences, as well as specific sequences that were distinctive of the most polluted samples, revealing patterns of functional diversity in this environment and its relation with environmental stressors. In addition, cloning of large metagenomic fragments from uncultured Planctomycetes allowed us to gain insight into possible novel features of this poorly known but ecologically relevant microbial group.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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