Alkane-degrading properties of *Dietzia* sp. H0B, a key player in the *Prestige* oil-spill biodegradation (NW-Spain)

Running headline: Alkane-degrading activity in *Dietzia* H0B

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Abstract

Aims: Investigation of the alkane-degrading properties of *Dietzia* sp. H0B, one of the isolated Corynebacterineae strains that became dominant after the *Prestige* oil spill.

Methods and Results: Using molecular and chemical analyses the alkane degrading properties of strain *Dietzia* sp. H0B were analyzed. This Gram-positive isolate was able to grow on *n*-alkanes ranging from C₁₂ to C₃₈ and branched alkanes (pristane and phytane). 8-hexadecene was detected as an intermedicate of hexadecane degradation by *Dietzia* H0B, suggesting a novel alkane-degrading pathway in this strain. Three putative alkane hydroxylase genes (one *alkB* homologue and two CYP153 gene homologues of cytochrome P450 family) were PCR-amplified from *Dietzia* H0B and differed from previously known hydroxylase genes, which might be related with the novel degrading activity observed on *Dietzia* H0B. The alkane degradation activity and the *alkB* and CYP153 gene expression were observed constitutively regardless of the presence of the substrate, suggesting additional, novel pathways for alkane degradation.

Conclusions: The results from this study suggest novel alkane-degrading pathways in *Dietzia* H0B and a genetic background coding for two different putative oil-degrading enzymes, which is mostly unexplored and worth to be subject of further functional analysis

Significance and Impact of the Study: This study increases the scarce information available about the genetic background of alkane degradation in genus *Dietzia* and suggests new pathways and novel expression mechanisms for alkane degradation.

Keywords: *Dietzia*, oil-spill, alkane degradation, constitutive expression, *Prestige*, CYP153, *AlkB*

Abbreviations: SCL, short-chain-length; MCL, medium-chain-length; LCL- long-chain-length; pAHs, particulate alkane hydroxylases; Cm, Chloramphenicol; HC, hydrocarbons; DGGE, Denaturing Gradient Gel Electrophoresis; SWsupp, Supplemented seawater; Pyr, Pyruvate; Hx, Hexadecane; DCM, dichloromethane; DAPI, 4'-6-Diamidino-2-phenylindole; DEPC, diethylpyrocarbonate; RT, retrotranscription; qPCR, Real-Time or quantitative PCR; aa, aminoacids; ALK, alkanes; Pri, Pristane (2,6,10,14-tetramethylpentadecane); Phy, Phytane (2,6,10,14-tetramethylhexadecane)

54 **Introduction**

55 Fuel oil-derived compounds are one of the most common pollutants in the oceans. Bioremediation,
56 defined as the act of adding materials to contaminated environments to accelerate the natural
57 biodegradation processes, is accepted as an appropriate oil spill response tool (Swannell *et al.*, 1996;
58 Venosa and Zhu 2003). Fuel oils are complex mixtures of four groups of compounds (aliphatics,
59 aromatics, resins and asphaltenes). The last two fractions are considered to be recalcitrant and
60 microbial catabolic pathways for the biodegradation of the former two have already been investigated
61 (Van Hamme *et al.*, 2003).

62 Information about the vast majority of indigenous marine bacteria is limited, and more efforts are
63 needed to isolate key oil-degrading bacteria (Teramoto *et al.*, 2009; Teramoto & Harayama, 2011), to
64 investigate their physiological requirements that may favour their growth after an oil spill (Harayama
65 *et al.*, 1999; Van Hamme *et al.*, 2003; Harayama *et al.*, 2004). Most studies have focused on the short-
66 term effects of oil spills on marine bacterial communities, which usually became dominated by
67 *Gammaproteobacteria* (e.g. *Pseudomonas* spp., *Alcanivorax* spp. etc.) after biostimulation
68 amendments (Macnaughton *et al.*, 1999; Roling *et al.*, 2002; Roling *et al.*, 2004). These groups of
69 bacteria are associated with a first fast petroleum degradation phase where Gram-positive bacteria are
70 never dominant. Most Gram-positive HC (hydrocarbon) degraders belong to the suborder
71 corynebacterineae including *Rhodococcus*, *Dietzia*, *Gordonia*, *Nocardia* and *Mycobacterium*.
72 Information about the role of the Gram-positive bacteria in degradation processes at oil polluted
73 environments is scarce, but many groups of the Gram-positive bacteria are known for their remarkable
74 metabolic and enzymatic versatility (Larkin *et al.*, 2005), suggesting the possibility of new diverse
75 enzymes toward complete biodegradation of the spilled oil. Although the ability to grow on medium-
76 chain-length (MCL) to long-chain-length (LCL) *n*-alkanes is a very common property of Gram-
77 negative as well as Gram-positive bacteria, only members of the former, such as *Pseudomonas putida*
78 GPo1 (van Beilen *et al.*, 2001) and more recently *Alcanivorax borkumensis* SK2 (Hara *et al.*, 2004;

79 Sabirova *et al.*, 2006), have been studied in depth (enzymology, genetics, and potential applications)
80 (van Beilen *et al.*, 2002; van Beilen and Funhoff 2007).

81 The most extensively characterized pathways for aerobic alkane-degradation are encoded by OCT
82 plasmids carried by Gram-negative bacteria like *Pseudomonas* (van Beilen *et al.*, 1992; van Beilen *et*
83 *al.*, 2001) where integral membrane non-heme diiron monooxygenases of the AlkB-type allow growth
84 on *n*-alkanes with carbon chain lengths from C₅ to C₁₆ (van Beilen and Funhoff 2007). These
85 particulate alkane hydroxylases (pAHs), encoded by *alkB* genes, are found as well in a wide range of
86 Actinomycetales containing Corynebacterineae. The pAHs require two soluble electron transfer
87 proteins, named rubredoxin and rubredoxin reductase, which transfer electrons to the active site of the
88 enzyme oxidizing alkanes to 1-alkanols. These products are then further metabolized by alcohol and
89 aldehyde dehydrogenases to fatty acids that enter the central metabolism via the β -oxidation pathway.
90 The other major group of enzymes related with terminal oxidation of MCL to LCL *n*-alkanes up to C₁₆
91 are cytochromes P450, mainly from family CYP153, which has been recently proposed as common in
92 alkane-degrading eubacteria lacking the integral membrane hydroxylases (van Beilen *et al.*, 2006).

93 Gram-positive Corynebacterineae bacteria, including *Dietzia* strains, dominated the *in situ*
94 bioremediation process of the stranded fuel after the *Prestige* oil-spill catastrophe (Alonso-Gutierrez *et*
95 *al.*, 2009) and *Dietzia* bacteria are frequently related to environments polluted with HC (Brito *et al.*,
96 2006; Kleinstauber *et al.*, 2006; von der Weid *et al.*, 2006). *Dietzia* strains are recognized as ideal
97 bacteria for enhancing HC-degrading activities of the surrounding bacteria increasing the hydrogenase
98 and catalase activities of other key oil-degrading species and therefore speeding up the biodegradation
99 process (Pleshakova *et al.*, 2008) and for their long term survival in the environment even under dry,
100 resource-limited conditions (Radwan *et al.*, ; Quatrini *et al.*, 2008; Alonso-Gutierrez *et al.*, 2009).

101 Bihari *et al.*, (2011) recently published the functional analysis of alkane degradation by *Dietzia* spp.
102 and revealed novel AlkB hydroxylases in this largely unexplored genus, which is therefore likely to
103 harbour many other interesting properties.

104 The present study reports on the alkane degrading properties and putative alkane degrading genes of
105 an autochthonous *Dietzia* strain, which appeared after the oil-spill catastrophe as a predominant
106 population (Alonso-Gutierrez *et al.*, 2009). The information derived from the present study will help in
107 understanding and monitoring the *in situ* activities of *Dietzia* bacteria at fuel oil polluted sites.

108

109 **Materials and methods**

110 **Bacterial strains**

111 Gram-positive Actinobacteria from suborder Corynebacterineae had been detected as a major,
112 naturally occurring group in the degradation of the *Prestige* oil accumulated at supralittoral rocky
113 shorelines of the Spanish coast 12 months after the accident (Alonso-Gutierrez *et al.*, 2009). One
114 strain, identified in that study as *Dietzia* spp. PDR22 (EU374995)), was renamed as *Dietzia* sp. strain
115 H0B and subjected to a deeper phylogenetic analysis based on an almost complete fragment of 16S
116 rRNA genes using primers F27 to R1492 as previously described (Alonso-Gutierrez *et al.*, 2008).

117

118 **Pregrowth condition**

119 To obtain sufficient cell population for alkane degradation assays, bacteria were cultured for 72 hours
120 on 50 mL supplemented seawater (SWsupp, hereafter) as previously described (Teramoto *et al.*, 2009).
121 Briefly, seawater was collected from the Pacific Ocean, 300 km off the coast of Tokyo (Japan) and
122 supplemented with 1g of NH_4NO_3 , 0.2 g of K_2HPO_4 , 12 mg of FeCl_3 and 0.1 g of yeast extract per liter
123 of seawater to which 0.5 % of a C source (namely pyruvate (Pyr) or hexadecane (Hx)) was added.

124

125 **Alkane degradation activity assay**

126 *Dietzia* H0B was compared with *Alcanivorax* SK2 for its ability to grow on different hydrocarbons
127 using Arabian crude oil as the only C source (Fig. 1). Briefly, cells of each bacterium were pre-grown
128 on Arabian crude oil at r.t. and collected at stationary phase. Then 50 μl of this culture was inoculated

129 into 3 ml sterilized SWsupp spiked with 3 μ l crude oil, and this culture was incubated up to 96 hours
130 with shaking (200 r.p.m. at 25°C). Hydrocarbons were extracted from the cultures every ~8 hours to
131 check the degree of oil degradation using gas chromatography-mass spectrometry (GC-MS) (Fig. 1).
132 Non-inoculated tubes were similarly incubated and served as controls.

133 HC were measured from the cultures as previously described (Teramoto *et al.*, 2009). Briefly,
134 samples were extracted twice with an equal volume of DCM. Sodium sulfate was added to dehydrate
135 the extracts, and the supernatants were concentrated to approximately 100 μ l by N₂ purging. The
136 concentrated extracts were subjected to GC-MS using an Agilent 6890A Gas chromatograph with an
137 Agilent 5973 mass selective detector (Agilent Technologies, USA) equipped with an Ultra 2 fused
138 silica capillary column (25 m in length, 0.2 mm in diameter and 0.33 mm in film thickness; HP
139 19091B-102, Agilent Technologies, USA). The temperatures of the injection port, transfer line, MS
140 source and quadrupole were maintained at 250, 250, 230 and 150°C, respectively. The column
141 temperature was increased from 80°C to 320°C at a rate of 5°C min⁻¹ for the first 48 min, and then kept
142 at 320°C for the next 20 min. Helium was used as the carrier gas at a constant pressure of 20 p.s.i. (138
143 kPa). All peak areas for HC obtained with the GC-MS selected ion monitoring were normalized by
144 dividing the peak area by that of 17a(H),21b(H)-hopane (Prince *et al.*, 1994).

145 In order to study if the alkane-degradation was constitutively expressed in *Dietzia* sp. H0B, cells
146 were pre-grown on (Pyr) and then tested for its ability to degrade alkanes (i.e. Hx) in the presence of
147 Chloramphenicol (Cm, an antibiotic which inhibits the synthesis of proteins at translation level).
148 Briefly, cells were harvested at the early stationary phase (10⁷-10⁸ cells ml⁻¹) and resuspended in 3 ml
149 of SWsupp to reach a high-density population (around 10¹⁰ cells ml⁻¹). This concentration (which is
150 approximately 1 g dry weight·L⁻¹) corresponds approximately to that of an outgrown shake flask
151 culture (two orders of magnitude bigger than that at stationary phase for *Dietzia* (10⁸, Fig. 1), leaving
152 very little room for increase in net biomass during the experiment. Then, 3 μ l of the tested alkane (Hx)
153 was added to the cell resuspension to reach a final concentration of 1000 p.p.m. Phenathrene (a

154 compound not degraded by the strains under study) and Cm were added at the same time that the Hx to
155 the high-density cell cultures at a final concentration of $0.25 \mu\text{g ml}^{-1}$ and 0.1 g L^{-1} , respectively
156 (*Dietzia* H0B was sensitive to this concentration of Cm; results not shown). The disappearance of the
157 initial compound and the appearance of potential intermediate molecules (e.g. hexadecanol,
158 hexadecanal, hexadecanoate, etc) were registered using GC-MS analysis after an incubation period of
159 2 days (at 200 r.p.m. and 25°C). The protocol for GC-MS analysis was as described above for Arabian
160 crude oil except that all HC peak areas were normalized by that of phenanthrene instead of
161 17a(H),21b(H)-hopane.

162

163 **DAPI counts**

164 Different dilutions, in sterile water, of the original culture were fixed in a final volume of 1ml of 4%
165 (v/v) paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries, Japan). Fixed cells
166 were stained using $1\mu\text{g ml}^{-1}$ of DAPI and filtered using polycarbonate black membrane filter (0.2 mm
167 pore size; Advantec Toyo, Ltd., Japan). The filters were examined under 1000X amplification with a
168 fluorescence microscope (Olympus IX81). Bacteria were attached to each other surrounding oil drops
169 making difficult an accurate measurement of the population. Cell counts were repeated at 10 randomly
170 selected fields to avoid as possible the heterogenous distribution of the filtered bacteria on the
171 membrane.

172

173 **Nucleic acid extraction**

174 Bacterial cells were boiled on sterile Milli-Q H_2O for 10 minutes to extract DNA from bacteria. For
175 RNA isolation, cells grown on 1000 p.p.m. of Pyr or Hx in SWsupp were collected at early stationary
176 phase (10^7 - $10^8 \text{ cells ml}^{-1}$) on DEPC cleaned tubes and homogenized with Trizol reagent (Invitrogen) as
177 previously described (Poisa-Beiro *et al.*, 2009). RNA was treated with Turbo-DNA free kit (Ambion)

178 to remove contaminating DNA. Final concentration and purity were measured using NanoDrop to
179 discard low quality extracted samples before further analysis.

180

181 **Analysis of *alkB* and *CYP153* genes**

182 PCRs were performed in a final volume of 50 µl containing 1.25 U of Taq (TaKaRa ExTaq™ Hot Start
183 Version; TaKaRa Bio Inc., Otsu, Siga, Japan), 1X ExTaq Buffer (2mM MgCl₂), 200 µM of each
184 deoxynucleoside triphosphate, 100 ng of template DNA and 0.5 µM of primers *alkB*1-f/-r (Kloos *et al.*, 2006) or P450-fw1/-rv3 (van Beilen *et al.*, 2005) for the amplification of *alkB* and *CYP153* gene
185 homologues, respectively (Table 1). The reaction mixtures were subjected to the following thermal
186 cycling parameters: (i) a hot start (9 min at 95°C); (ii) 30 cycles, with one cycle consisting of 30
187 seconds at 96°C for denaturation, 30 seconds at 58°C for annealing, and 1 min at 72°C for elongation;
188 and (iii) a final extension step of 10 min at 72°C. The thermal profile used for *CYP153* genes was
189 exactly the same but, in order to avoid appearance of unspecific bands, a touchdown thermal profile
190 protocol was carried out during the first 10 cycles of step (ii), where the annealing temperature was
191 decreased by 1°C per cycle from 65°C to 60°C followed by 20 additional cycles at 60°C of annealing
192 temperature. These cycles were performed with a Perkin-Elmer GeneAmp 2700 Thermocycler
193 (Applied Biosystems, Foster City, CA, USA).

195 PCR products were separated by electrophoresis in a 1% (w/v) agarose gel in 1×TAE buffer stained
196 with ethidium bromide and photographed immediately under UV light using Gel Doc XR system and
197 Quantity One software (Bio-Rad, Hercules, CA). 100 and 50 bp ladders were used as size markers
198 (Invitrogen).

199 Those PCR products with the expected size (around 550 bp and 350 bp for *alkB* and *CYP153* genes
200 respectively), were cloned into pCR®2.1-TOPO® vector and transformed into competent *Escherichia*
201 *coli* TOP 10F' cells, following manufacturer's protocol of TOPO T/A Cloning Kit (Invitrogen).
202 Screening of clones with insert of the correct size was performed using primers M13F and M13R.

203 Plasmid DNA (of 10 transgenic clones per gene and strain) was isolated as previously described
204 (Maniatis *et al.*, 1982).

205

206 **Sequencing and phylogenetic analyses**

207 Excess of primers and dNTPs was removed by enzyme digestion using 10 and 1U of ExoI and SAP,
208 respectively (Amersham Biosciences) at 37°C for 1 hour followed by inactivation at 80°C for 15 min.

209 Sequencing was accomplished using the ABI PRISM™ Big Dye Terminator Cycle Sequencing
210 Ready Reaction Kit (version 3.1) and an ABI PRISM™ 3700 automated sequencer (PE Applied
211 Biosystems, Foster City, California, USA) following the manufacturer's instructions. Cloned *alkB* and
212 *CYP153* genes' fragments were sequenced in both directions using vector primers M13F and M13R.
213 The sequences were inspected for the presence of ambiguous base assignments, hand edited using Edit
214 View (Applied Biosystems) and FinchTV (Geospiza Inc.) and finally assembled using GeneJockeyII
215 (Taylor P.L. 1995, BIOSOFT) into a single consensus sequence. After that, the sequences were
216 examined with the BLAST search alignment tool comparison software (BLASTN, BLASTx and
217 tBLASTx) (Altschul *et al.*, 1990) to detect the closest bacterial sequences within the GenBank
218 database.

219 Amino acid sequences were inferred from nucleotide sequence data using the EMBOSS: Transeq
220 program of EMBL-EBI (European Bioinformatics Institute, <http://www.ebi.ac.uk/>) and aligned using
221 MAFFT (<http://align.bmr.kyushu-u.ac.jp/mafft/online/server/>) (Katoh and Toh 2008) with reference
222 ones obtained from GenBank. The alignment obtained was edited in MacClade program (Maddison
223 and Maddison 2003) and directly transferred to version 4.0b10 of PAUP*software (Swofford 2000).
224 ProtTest software version 10.2 (Abascal *et al.*, 2005) was run as a guide to determine the best-fit
225 maximum likelihood (ML) model for the edited alignment. ProtTest examines models of protein
226 evolution like Blosum62, Dayhoff, JTT, etc. The best-fit models of protein evolution, calculated by
227 ProtTest were incorporated into software PHYML (Guindon and Gascuel 2003), which uses a single,

fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood. Finally, the trees created by PHYML were edited using the software TreeViewX (Page 1996).

The *alkB* and CYP153 expression studies

Expression of *alkB* and CYP153 genes isolated from *Dietzia* H0B was assessed by qPCR. RNA from Hx- and Pyr-grown bacteria was isolated as described above and five µg of each were used to obtain cDNA using the Superscript II Reverse Transcriptase and random primers (Invitrogen) following the manufacturer's instructions. qPCR assays were performed using the 7300 Real Time PCR System (Applied Biosystems) with specific primers (Table 1). The 0.5 µl of 10 µM of each primer and 1 µl of the cDNA template were mixed with 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 µl. The standard cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C 15 s and 60 °C 1 min. The comparative CT method ($2^{-\Delta\Delta C_t}$ method) was used to determine the expression level of analyzed genes (Livak and Schmittgen 2001). The expression of the target genes, *alkB* and CYP153 genes, was normalized using primer pair DNApolIV fw/rv (Sharp *et al.*, 2007; Table 1), able to amplify the housekeeping gene coding for the DNA polymerase IV (DNApolIV), which were constitutively expressed in our strain (H0B) irrespective of the substrate used. Fold units were calculated dividing the normalized expression values of Hx-grown cells by the normalized expression values of the Pyr-grown cells. Strain cultivation, RNA extraction and RT-PCR were performed twice individually. For each gene and strain, three different q-PCR readings were performed.

Nucleotide sequence accession numbers

The nucleotide sequences identified and used in this study have been deposited in the GenBank database under accession numbers: FJ435349 and FJ435352 (16S); FJ435353 and FJ435355 (*alkB*); and FJ435356, FJ435360 and FJ435362 (CYP153).

Results

Morphology and phylogenetic analysis

From the microscope observations *Dietzia* H0B appeared as coccoid- to ovoid-shaped cells that, similarly to *Alcanivorax* cells, tended to grow attached to oil surface forming dense granules around drops showing its high hydrophobicity (results not shown).

The almost full 16S rRNA gene sequence of *Dietzia* H0B was quite similar to that of *Dietzia psychralkaliphila* (99-100%;) (Fig. 2A), previously described as an *n*- and branched alkane degrading bacterium (Yumoto *et al.*, 2002).

Petroleum-hydrocarbon degrading abilities of *Dietzia* H0B

Cell number of *Dietzia* H0B did not increase so much in response to high concentrations of crude oil in contrast with *Alcanivorax* SK2 (Fig. 1), which is known to increase rapidly under high HC inputs (Kasai *et al.*, 2002; Alonso-Gutierrez *et al.*, 2008). Doubling times of 2-3 and 5-6 hours were calculated for *Alcanivorax* SK2 and *Dietzia* H0B respectively (Fig.1), which are close to previously reported values (Binazadeh, *et al.*, 2009; Teramoto *et al.*, 2009). This opposing growth behaviour between Gram-positive and Gram-negative bacteria has been previously observed (Margesin *et al.*, 2003).

Dietzia H0B was able to grow on *n*-alkanes ranging from C₁₂ to C₃₈ from Arabian crude oil and also branched alkanes (pristane and phytane) (Fig. 1). Yumoto *et al.* (2002) also observed growth of a *Dietzia* strain with pristane.

Dietzia H0B produced 8-hexadecene when growing on Hx as suggested by the MS data. The normalized peak area of the 8-hexadecene detected after 48 hours accounted for a 1% of the peak area of the hexadecane added.

Detection of putative genes coding for AlkB and for cytochrome P450 of CYP153 family

278 Putative genes coding for alkane-degrading enzymes, alkane hydroxylase AlkB and cytochrome P450
279 of CYP153 family, were detected in *Dietzia* H0B and were compared with their closest sequences
280 from Gram-positive isolates (McLeod *et al.*, 2006; Stinear *et al.*, 2008) or uncultured bacteria (Kubota
281 *et al.*, 2005) (Fig. 2 B, C). The phylogenetic analysis revealed that putative AlkB coding genes from
282 *Dietzia* H0B differed from any previously described *alkB* (Fig. 2B).

283 CYP153 cytochromes from *Dietzia* H0B were close to sequences derived from metagenomic
284 studies of petroleum-contaminated sites (e.g. BAE47478-81(Kubota *et al.*, 2005) in Fig. 2C). It is also
285 interesting that the CYP153 genes isolated from *Dietzia* H0B (CYP153_H0B_uniq and
286 CYP153_H0B_C4) were quite different from each other (Fig. 2C). DNA sequences have 247 out of
287 294 b.p. in common meaning only 84% of identity between them. Bootstrap values were always low
288 due to the small size of the sequence (98 aa) analyzed and the high variability of these enzymes among
289 bacteria.

290

291 **Constitutive expression of alkane degradation activity**

292 Alkane-degrading assays showed that degradation of hexadecane in Pyr-pre-grown cells of *Dietzia*
293 H0B in the presence of chloramphenicol (Cm) was possible, suggesting that the alkane degrading
294 enzyme(s) was constantly expressed in this strain. *Dietzia* H0B was pregrown on Pyr and then
295 incubated with Hx and Cm for up to 2 days. The presence of Cm inhibited protein expression during
296 the degrading assay and therefore no degradation would be possible unless the alkane-degrading
297 enzymes would have been previously expressed during its growth on Pyr. The amount of Hx in
298 cultures of Pyr-pre-grown cells was significantly lower than that in the control culture without
299 inoculation of the strain (C) as measured by Student's t-Test (Fig. 4), indicating that hydroxylases
300 were already expressed during its growth on Pyr.

301 In accordance with the activity assay, no significant difference was observed for the *alkB* and
302 CYP153 genes' expression levels between the Pyr- and Hx-grown cells of *Dietzia* H0B (Fig. 5). These

303 data support the constitutive expression of alkane degrading activity in *Dietzia* H0B.

304

305 **Discussion**

306 In the present study, we investigated the alkane-degrading abilities of *Dietzia* H0B. This Gram-
307 positive strain was selected among the autochthonous Corynebacterineae bacteria that dominated the
308 alkane-degrading microbial community on oiled sand and rock matrices 12 months after the *Prestige*
309 oil-spill catastrophe (Alonso-Gutierrez *et al.*, 2009).

310 Although most studies reveal that oil-contaminated sites are dominated by Gram-negative bacteria
311 (Mac-Naughton *et al.*, 1999; Roling *et al.*, 2004), the dominant fraction of HC degraders of this site
312 was composed of GC-rich mycolic acid containing Gram-positive Actinomycetes of the genera
313 *Rhodococcus*, *Mycobacterium* and *Dietzia* (Alonso-Gutierrez *et al.*, 2009). All these genera are
314 recognized as ideal candidates for the biodegradation of HCs (Larkin *et al.*, 2005; Kloos *et al.*, 2006).
315 *Actinomycetes* bacteria have been previously suggested to play key roles in bioremediation of *n*-
316 alkane-contaminated sites under dry, resource-limited conditions in marine shorelines (Quatrini *et al.*,
317 2008, Alonso-Gutierrez *et al.*, 2009). However, there are very few studies about their degrading
318 properties and only until very recently the genetic background of *Dietzia* has been subject of study
319 (Bihari *et al.*, 2011).

320 The degradation of more recalcitrant HC, LCL *n*-alkanes and branched alkanes, showed by *Dietzia*
321 H0B, has been reported from only few genera including *Alcanivorax* and *Rhodococcus* (Harayama *et*
322 *al.*, 1999; Harayama *et al.*, 2004). The combination of microscopy, alkane degradation assays and gene
323 expression analyses used in this study suggests high hydrophobicity and diverse putative alkane-
324 degrading genes, pathways and expression patterns in *Dietzia* H0B. All these characteristics might
325 explain the dominance of *Dietzia* and other Actinobacteria in the long-term after the Prestige oil-spill
326 under resource-limited conditions.

327 The high hydrophobicity developed by Actinobacteria might be explained by the outer permeability

328 barrier made of mycolic acids (very-long-chain (C₃₀–C₉₀) α -alkyl, β -hydroxy fatty acids), which are
329 major and specific constituents of Corynebacterineae (Gebhardt *et al.*, 2007) and so is in *Dietzia* spp.
330 (Yumoto *et al.*, 2002). This feature has been previously related to its enhanced biodegradation capacity
331 (Linos *et al.*, 2000; Lee *et al.*, 2006) and the explanation for its preferential access to the most
332 recalcitrant fractions of the fuel (van Hamme *et al.*, 2001, Alonso-Gutierrez *et al.*, 2009).

333 Both *alkB* and CYP153 coding genes isolated from *Dietzia* H0B were novel (Fig. 2 B, C),
334 supporting the enzymatic versatility of this genus. *AlkB* and CYP153, which are found with high
335 diversity among Gram-positive bacteria, are structurally distant enzymes (Heiss-Blanquet *et al.*, 2005;
336 van Beilen and Funhoff 2005; van Beilen *et al.*, 2006). The functional meaning of multiple alkane
337 hydroxylase systems is unknown and could be related to the oxidation of a different range of alkanes
338 or the level of activity during different growth phases (Marin *et al.*, 2003). Accordingly,
339 CYP153_H0B_uniq (Fig. 2C) might code for enzyme with the unique degrading ability observed in
340 the strain.

341 Interestingly, GC-MS suggested that *Dietzia* H0B produced 8-hexadecene, when growing on Hx.
342 Although in small quantities this alkene was always detected when *Dietzia* H0B was grown on
343 hexadecane, suggesting that the compound might be an intermediary compound of the degrading
344 pathway. To our knowledge, this conversion has not been previously observed in any other alkane
345 degrading bacteria. This could indicate a novel degrading pathway in this species which start the
346 oxidation process at the middle of the alkane chain instead of doing it at terminal or
347 subterminal positions as previously described (van Beilen and Funhoff 2007).

348 Expression of alkane degrading enzymes in bacteria including *Pseudomonas*, *Alcanivorax* (Gram-
349 negative (Yuste *et al.*, 1998; Sabirova *et al.*, 2006)) and *Rhodococcus* (Gram-positive;(Bredholt *et al.*,
350 2002)) bacteria is induced by alkanes. However, alkane-degrading activity in *Dietzia* H0B is expressed
351 in the absence of alkanes (Fig. 3). In addition, no significant difference was observed for the *alkB* and
352 CYP153 genes' expression levels between the Pyr- and Hx-grown cells of *Dietzia* H0B (Fig. 4). These

353 data support for the constitutive expression of alkane degrading activity in *Dietzia* H0B, which might
354 be considered as a disadvantage since maintaining enzymes while their substrates are not present
355 would waste energy and resources. However, one plausible explanation is that the genes explored (one
356 AlkB and two CYP153) are not the only genes responsible for the degradation of alkanes. There could
357 be some other more relevant genes apart from those selected that might be induced in the presence of
358 alkanes and/or explain the presence of hexadecene as a novel intermediate. In fact, even in the
359 presence of chloramphenicol, we would expect an almost complete degradation of the alkanes after 2
360 days of incubation if all the hydroxylases were constitutively expressed. In this sense, a recent study
361 has shown that only four out of the nine putative genes isolated from an oil-degrading strain of *Dietzia*
362 were strongly induced by long-chain *n*-alkanes (Bihari *et al.*, 2011). Most interestingly, one of these
363 sequences encoded a natural fusion protein consisting of an integral membrane alkane hydroxylase and
364 a rubredoxin domain, which plays a major role in long-chain *n*-alkane degradation. The results from
365 this study evidence the functional and catabolic diversity of genus *Dietzia* spp. and the presence of
366 novel degrading enzymes which might escape detection using previously described primers.

367 Even though the constitutive alkane degrading activity reported in the present study might
368 correspond only to some of their hydroxylases, this observation could be in agreement with previous *in*
369 *situ* observations where Gram-positive populations do not fluctuate in response to the increase of HC
370 from an oil spill in contrast with Gram-negative bacteria (Margesin *et al.*, 2003). Margesin *et al.*,
371 (2003) observed a highly significant positive correlation ($P < 0.001$) between the level of
372 contamination and the number of genotypes containing genes from *P. putida* and *Acinetobacter* sp.
373 (Gram-negative) but no significant correlation between the HC content and the number of genotypes
374 containing genes from Gram-positive bacteria (*Rhodococcus alkB1* and *alkB2* and *Mycobacterium*
375 *nidA*), suggesting that these genes were not induced by the increasing concentration of alkanes.

The constitutive expression of some of the putative alkane degrading genes detected in *Dietzia* could be critical for the complete degradation of the most recalcitrant fractions in the long term after an oil-spill, when new inputs of HC are no longer available but activity needs to be maintained.

In conclusion, this work provide a glimpse of the great catabolic diversity of indigenous Gram-positive bacteria and point out the need for further research on Actinobacteria, specially Corynebacterineae, previously suggested as key players in the degradation of recalcitrant fractions due to its special cell wall and catabolic properties (Quatrini *et al.*, 2008; Alonso-Gutierrez *et al.*, 2009). Our results shed light on the genetics and physiology of autochthonous Corynebacterineae (genus *Dietzia*), which was supposed to constitutively express diverse catabolic enzymes and new alkane degradation pathways that might be important for the complete degradation of fuel-oil hydrocarbons. Besides, the primers and sequences derived from this study can be used for the *in situ* monitoring of these Corynebacterineae bacteria in the long-term bioremediation processes after an oil-spill.

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566 **Table 1** Primers used in this study

Primer	Sequence (5'-- 3')	Bases number	Product size	Reference
alkB-1f	AAYACNGCNCAYGARCTNNGNCAYAA	26	550 bp	(Kloos <i>et</i>
alkB-1r	GCRTGRTGRTCNGARTGNCGYTG	23	(approx)	<i>al.</i> , 2006)
CYP153 (P450fw1)	GTSGGCGGCAACGACACSAC	20	339 bp	(van Beilen
CYP153 (P450rv3)	GCASCGGTGGATGCCGAAGCCRAA	24	(approx)	<i>et al.</i> , 2005)
DNA pol IV fw	GACAACAAGTTACGAGCCAAGATC	24	27 bp	(Sharp <i>et</i>
DNA pol IV rv	CCTCCGTCAGCCGGTAGAT	19	(approx)	<i>al.</i> , 2007)
AlkB_H0B_fw	CTGATCGTCCAGGCAATCTT	20		
AlkB_H0B_rv	GCTGCAGGTGGTACAGGAAG	20	172 bp	This study
CYP153_H0B_C4_f				
w	CGAGAAGCTCAAGTCCAACC	20		
CYP153_H0B_C4_r				
v	CACATCACGACCTTGTCACC	20	153 bp	This study
CYP153_H0B_UN_f				
w	CCAGTTCGAGAAGCTCAAGG	20		
CYP153_H0B_UN_r				
v	CTGACGCATACCACATGACC	20	170 bp	This study

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582 **Figure legends**

583 **Figure 1** Growth curve (open symbols) and degradation of n-alkanes (black-filled symbols)
584 and branched alkanes (grey-filled symbols) by the alkane-degrading *Dietzia* H0B (squares)
585 compared to *Alcanivorax* strain SK2 (triangles). 150 µl of bacterial culture at late exponential
586 growth was inoculated into 3 ml SWsupp medium containing 3 µl crude oil, and the culture
587 was incubated for the indicated period at 25 °C. Non-inoculated sterile samples were similarly
588 incubated and served as controls (100 %). Oil was extracted from the cultures as described
589 in Methods. The *n*-alkanes (C_{12–22}; black-filled symbols) and branched alkanes (pristane and
590 phytane; grey-filled symbols) were quantified. The degradation and growth curve of is also
591 shown as a reference.

592

593 **Figure 2** Phylogenetic analysis of gene sequences detected in *Dietzia* H0B. (a) Phylogenies
594 based on almost complete 16S rRNA gene sequences (~1500bp) from the strain with those
595 of *Rhodococcus* and *Dietzia* type strains are shown. (b) Phylogenetic analysis of the deduced
596 amino acid sequence (166 aa) of the putative AlkB gene from *Dietzia* H0B. (c) Phylogenetic
597 analysis of the deduced amino acid sequence (98 aa) of the putative CYP153 coding genes
598 from *Dietzia* H0B. All phylogenies were estimated by maximum likelihood using the Akaike
599 information criterion (AIC) (Model of Nucleotide evolution: WAG+G; Pinvar: 0.06-0.08).
600 Bootstrap values ≥ 50 are shown at branch nodes (1000 iterations). Accession numbers of
601 reference sequences are indicated in parentheses while bold characters indicate sequences
602 derived from this study. *Thermotoga* sp. 16S gene, Xylene monooxygenase from
603 *Pseudomonas putida* and different families of cytochromes P450 related to CYP153 were
604 used as outgroup for the 16S (a), alkB (b) and CYP153 (c) phylogenies, respectively.

605

606 **Figure 3** Alkane degrading activity of *Dietzia* H0B pregrown on pyruvate (H0B-Pyr) in the
607 presence of chloramphenicol. Hexadecane (Hx) was added to the H0B-Pyr cells at 1000
608 p.p.m and this cell suspension was incubated for 2 days. Non-inoculated sterile samples
609 were similarly incubated and served as controls (100%). The amount of Hx that remained
610 after 2 days of incubation with H0B-Pyr-was significantly lower (*) than that of the control as
611 measured by Student's t-Test. Control samples for 10-minute incubation were prepared in
612 the same way with a naphthalene-degrading strain RP3 of a *Citricella* bacterium, unable to
613 degrade alkanes (100%). Standard deviations between the two replicates are indicated at top
614 of the bars.

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616 **Figure 4** Expression analysis using RT-qPCR of putative *alkB* and CYP153 genes from
617 *Dietzia* H0B grown on 1000 p.p.m. of Hx or Pyr to an early stationary phase (10^7 - 10^8 cells ml⁻¹). Bars represent fold induction of each gene expressed in the presence of Hx relative to the
618 expression level on Pyr. No significant differences were observed for any of the *alkB* and
619 CYP153 genes analyzed in *Dietzia* H0B. Expression levels were previously normalized using
620 a housekeeping gene (DNAPolIV). Standard deviations between the two replicates are
621 indicated at top of the bars.

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627 **Figure 1**

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Figure 2

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640 **Figure 3**

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653 **Figure 4**

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