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Bacterial Communities from Shoreline Environments (Costa da Morte, Northwestern Spain) Affected by the *Prestige* Oil Spill▽†

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The bacterial communities in two different shoreline matrices, rocks and sand, from the Costa da Morte, northwestern Spain, were investigated 12 months after being affected by the *Prestige* oil spill. Culture-based and culture-independent approaches were used to compare the bacterial diversity present in these environments with that at a nonoiled site. A long-term effect of fuel on the microbial communities in the oiled sand and rock was suggested by the higher proportion of alkane and polyaromatic hydrocarbon (PAH) degraders and the differences in denaturing gradient gel electrophoresis patterns compared with those of the reference site. Members of the classes *Alphaproteobacteria* and *Actinobacteria* were the prevailing groups of bacteria detected in both matrices, although the sand bacterial community exhibited higher species richness than the rock bacterial community did. Culture-dependent and -independent approaches suggested that the genus *Rhodococcus* could play a key role in the in situ degradation of the alkane fraction of the *Prestige* fuel together with other members of the suborder *Corynebacterineae*. Moreover, other members of this suborder, such as *Mycobacterium* spp., together with *Sphingomonadaceae* bacteria (mainly *Lutibacterium anuloederans*), were related as well to the degradation of the aromatic fraction of the *Prestige* fuel. The multiapproach methodology applied in the present study allowed us to assess the complexity of autochthonous microbial communities related to the degradation of heavy fuel from the *Prestige* and to isolate some of their components for a further physiological study. Since several *Corynebacterineae* members related to the degradation of alkanes and PAHs were frequently detected in this and other supralittoral environments affected by the *Prestige* oil spill along the northwestern Spanish coast, the addition of mycolic acids to bioremediation amendments is proposed to favor the presence of these degraders in long-term fuel pollution-affected areas with similar characteristics.

Since the *Polycommander* accident, many other oil spills, such as the *Urquiola* (1976), *Andros Patria* (1978), and *Aegean Sea* (1992) spills, have occurred on the Galician coast (northwestern Spain), where intense maritime traffic takes place. On 13 November 2002, the oil tanker *Prestige* sprang a leak off Cape Finisterre (Galicia, northwestern Spain) and 6 days later its oil tank broke up and sank 240 km west of Galicia. The spill of 60,000 tons of heavy fuel oil polluted 500 miles of the Spanish coast, reaching the French coast. The Costa da Morte, northwestern Spain, was the most affected area (2). The oil residue released by the *Prestige* was devoid of the more labile fractions (boiling point, <300°C), with high levels of aromatic hydrocarbons (~50%), as well as resins and asphaltenes (~30%) (6).

Information about the autochthonous microbial populations at maritime oil-polluted sites is scarce (23). The studies carried out after the *Nakhodka* spilled oil with a chemical composition similar to that of the *Prestige* fuel, gathered information on the marine microbial populations that adapted to heavy fuel oil. Different molecular approaches, mainly involving 16S rRNA gene analyses such as PCR-denaturing gradient gel electrophoresis (DGGE) (31), clone libraries, and specific oligonucleotide probes (43), were used to describe the bacterial communities established in different environments and at different time intervals after the oil spill.

Most of the previous studies were focused either on the isolation of a few culturable degrading strains or just on detecting the 16S rRNA gene sequences of all of the bacteria present in polluted samples without gathering information on their physiology. As a consequence, more efforts should be made to understand community structures in situ and to isolate the key oil-degrading species present, with the aim to further investigate their requirements (23, 58) that could be used in the development of new bioremediation.

In the present report, we describe a microbiological analysis of a cobblestone beach on the Costa da Morte, northwestern Spain, affected by the *Prestige* heavy fuel oil spill 12 months after the last fuel stranding. The microbial community was examined thoroughly by a triple-approach method based on different cultivation strategies and culture-independent methods such as DGGE and the screening of 16S rRNA gene clone libraries.

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MATERIALS AND METHODS

Sampling. In March 2004, 12 months after the last fresh fuel stranding from the *Prestige* (Fig. 1), oil-polluted samples were taken from the supralittoral zone of a cobblestone beach located next to Faro Lariño (42°46′25″N, 09°07′30″W,

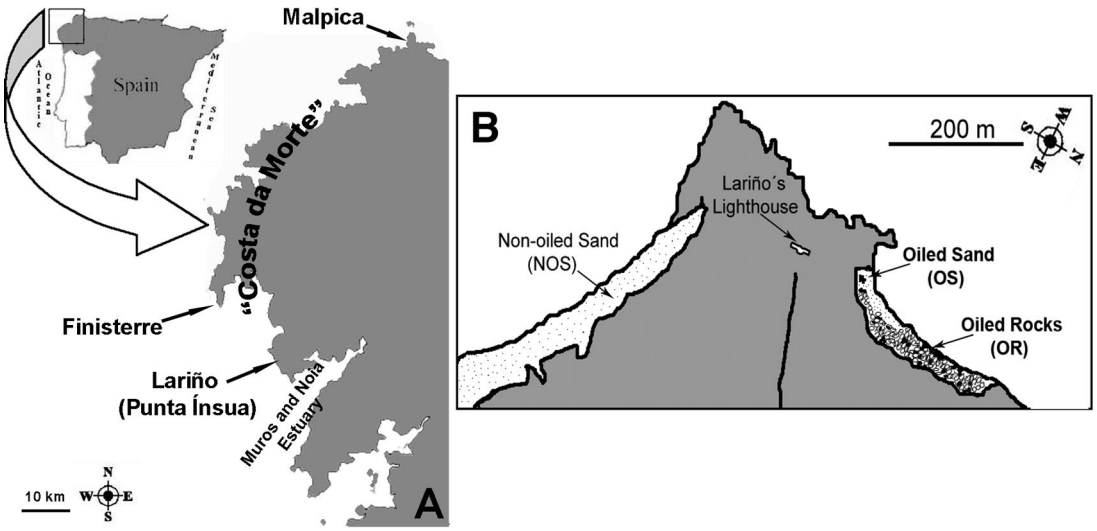

FIG. 1. (A) Detailed map of the northwestern coast of Spain (Galicia) known as the Costa da Morte or the Costa de la Muerte (from the Muros and Noia Estuary to Malpica), which means Coast of Death, for its strong swell and harsh weather. Punta Ínsua is the main landmark next to the sampling site. (B) Closer image of the sampling site.

Carnota, Spain). Samples included small oil drops scattered among sand grains (OS) and fuel paste attached to rock surfaces (OR). The heavy fuel from the *Prestige* attached to rock surfaces and interstices formed thick oil layers where different materials get attached. Nonoiled sand (NOS) samples from an adjacent zone were taken as controls. Samples were placed in sterilized glass jars and kept cool (4°C) or frozen (−20°C) until analysis (Fig. 2).

Chemical analysis. To assess the degree of biodegradation of the sample and to verify that no cross contamination from sources other than the *Prestige* fuel occurred, oil residues (1 g) were dissolved in 5.0 ml of dichloromethane (Supra-Solv grade; Merck, Darmstadt, Germany), phase separated, and percolated through 2 g of anhydrous sodium sulfate. The organic extracts were carefully evaporated until dried, and one aliquot (5 to 10 mg) was dissolved in hexane and then fractionated in a previously conditioned (with 6 ml hexane; Merck) cyanopropyl silica solid-phase cartridge (SiO_2/CN, 1.0/0.5 g, 6 ml; Interchim, Montluçon, France) as reported elsewhere (6). The aliphatic and aromatic fractions were obtained by elution with 4.0 ml of hexane (fraction 1) and 5.0 ml of hexane-dichloromethane (1:1) (fraction 2), respectively. Both fractions were then analyzed by gas chromatography-mass spectrometry on a TRACE-MS Thermo Finningan TRACE-GC 2000 gas chromatograph (Thermo Finningan; Dreieich, Germany) fitted with an HP 5MS (30 m by 0.25 mm [inside diameter] by 0.25 μm film) capillary column (J&W Scientific, Folsom, CA).

The extent of biodegradation of each compound was measured from the normalized peak area of the target analyte referred to that obtained from the same compound in the control sample (2, 28). The peak areas of the target analytes were measured in the reconstructed ion chromatograms at *m*/*z* 85 for aliphatics and at the corresponding molecular ion for the aromatics as described elsewhere (16).

Microbial characterization. Samples were analyzed directly (OR, OS) or enriched first with different fuel components such as alkanes or aromatics. The procedures and nomenclature used are summarized in Fig. 2 and Table 1, respectively.

Enumeration of heterotrophic and hydrocarbon-degrading microbial populations. Bacterial counts of heterotrophs and *n*-hexadecane and polyaromatic hydrocarbon (PAH) degraders were performed by a miniaturized most-probable-number (MPN) method in 96-well microtiter plates with eight replicate wells per dilution as described elsewhere (3, 69). All of the media used (tryptic soy broth and mineral medium BMTM [3]) were corrected to reach 3% NaCl. MPN analysis results are shown as means of triplicates, and the Student *t* test was used to compare them (Fig. 3).

Isolation of culturable strains. Culturable microorganisms from OR (rock) and OS (sand) samples and from enrichment cultures grown on phenanthrene or *n*-hexadecane were isolated onto different media (Table 1). Culturable heterotrophs were isolated at 20°C onto fivefold-diluted marine agar (MA 1/5) supplemented to maintain 3% NaCl. *n*-Hexadecane and phenanthrene degraders were isolated on mineral agar (BMTM agar, 3% NaCl) supplemented with *n*-hexadecane in the vapor phase (54) or phenanthrene (0.1%) as a sole carbon and energy source, respectively (Table 1). All isolated strains were stored at −80°C in 20% (vol/vol) glycerol for subsequent analysis.

Screening of the hydrocarbon-degrading capability of strains. All of the isolated strains were screened for the ability to degrade alkanes and aromatics on either solid or liquid mineral medium as previously described (3).

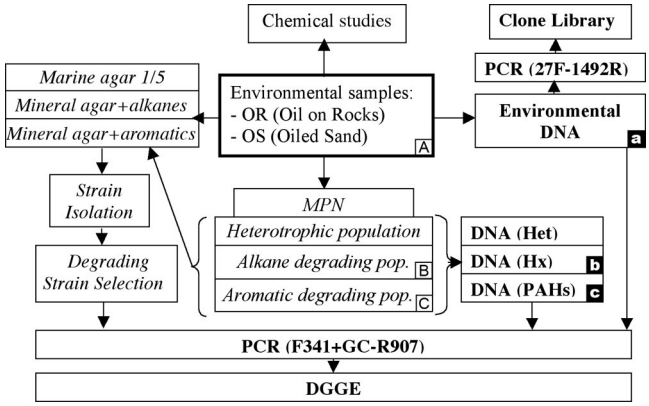

FIG. 2. Flow chart diagram illustrating the protocols used in this study. For both sample types (OR and OS), chemical, microbiological, and molecular analyses were done. Templates for PCR-DGGE were DNA extracted directly from the environment (samples A/DNAs a, in boxes), from the trophic populations (pop.) grown in MPN analysis plates (samples B and C/DNAs b and c, in boxes), or from degrading strains. The DGGE profiles of the total DNA of each sample and of its trophic populations (heterotrophic and alkane and aromatic degrading) were compared to identify common bands between them. Degrading strains isolated with different media were individually screened by DGGE to detect those that comigrated with specific bands in the profiles. A nearly full-length 16S rRNA gene PCR fragment from the total DNA (a) of the samples was cloned. The clone library was screened by restriction fragment length polymorphism analysis, and different operational taxonomic units were sequenced. Sequences from the most interesting bands and strains were compared with those from the clone library to determine the quantitative proportions of the different species found.

TABLE 1. Nomenclature of sequences retrieved in this study sorted by their methodological sources and origins as shown in the flow chart diagram in Fig. 1[a]

Sample and source (code)	OR samples			OS samples		
	Sequence code	Table(s) in supplemental material	Figure(s)	Sequence code	Table(s) in supplemental material	Figure(s)
DGGE bands from PCR-amplified:						
Total DNA (a)	R + n^{er}	S2	4A, 6A	S + n^{er}	S2	4A, 6B
DNA from MPN Hx (b)	RH + n^{er}	S3	4B, 6A	SH + n^{er}	S3	4C, 6B
DNA from MPN PAHs (c)	RPb + n^{er}	S3	4B, 6C	SP + n^{er}	S3	4C, 6C
Clone library from PCR-amplified total DNA (a)	Rc + n^{er}	S4		Sc + n^{er}	S4	
Strains isolated with:						
MA 1/5 from original samples (A)	RP + n^{er}	S1, S5		AP + n^{er}	S1, S6	
MA 1/5 from highest dilution in Hx MPN analysis (B)	RPH + n^{er}	S1, S5		APH + n^{er}	S1, S6	
MA 1/5 from highest dilutions in PAH MPN analysis (C)	RPP + n^{er}	S1, S5		APP + n^{er}	S1, S6	
BMTM + Hx from original samples (A)	PDR + n^{er}	S1, S5	6A	PDA + n^{er}	S1, S6	6B
BMTM + Phe from original samples (A)	PhR + n^{er}	S1, S5	6C	PhS + n^{er}	S1, S6	6C

[a] Figures and tables in the supplemental material related to Fig. 1 samples are indicated for clarity. MA, marine agar; BMTM, mineral agar; Phe, phenanthrene; Hx, *n*-hexadecane; Hx MPN or PAH MPN, plate counting of MPN of microbial populations related to alkane or aromatic degradation, respectively. A, B, and C, sample codes indicated in the flow chart diagram in Fig. 2. a, b, and c, DNA extracted from A, B, and C samples, respectively, as shown in Fig. 1.

To assess hydrocarbon-degrading capability in solid medium, mineral agar supplemented with *n*-hexadecane and phenanthrene was used as described above. Microtiter plates containing 200 μl per well mineral medium (BMTM, 3% NaCl) and *n*-hexadecane, F1, or a PAH mixture were used, as for MPN analysis, in liquid screenings. F1 is the aliphatic fraction (2.5 g · $liter^{-1}$) obtained from Casablanca crude oil (61).

To inoculate the biodegradation assays, the strains were grown overnight at room temperature on tryptic soy broth (3% NaCl). Cells were harvested by centrifugation at 4,000 × *g* for 15 min, washed twice, and finally suspended in mineral medium (BMTM plus 3% NaCl) to reach an optical density of 0.5 (determined at 620 nm with a Multiskan spectrophotometer [Labsystems]). Twenty microliters of suspended cells was used for the inoculation of two wells per plate. Another plate with only mineral medium was inoculated as a negative control. Only those wells with evident turbidity compared to the control plate were considered positive.

DNA extraction. Total community DNA was extracted from OR and OS samples by a bead-beating protocol with a PowerSoil DNA soil extraction kit (MoBio Laboratories, Inc., Solano Beach, CA) by following the manufacturer's instructions.

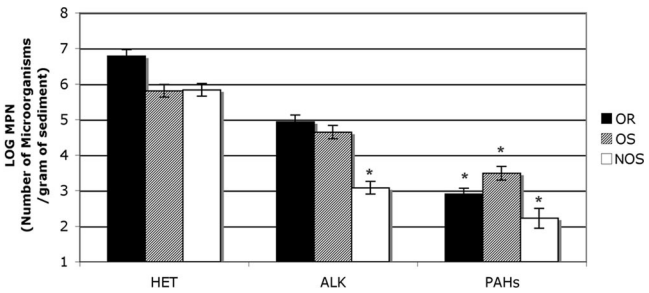

FIG. 3. MPN analysis of heterotrophic (HET), alkane-degrading (ALK), and PAH-degrading populations (PAHs) in polluted samples of rocks (OR) and sand (OS) compared with those in nonoiled sand (NOS). Standard deviations ($n = 8$) are represented by error bars. *, significantly different from the other two samples by the Student *t* test. Polluted samples have similar populations, except for aromatic degraders, while unpolluted samples always have significantly smaller degrading populations than polluted ones do.

Genomic DNA from the heterotrophic population and from those related to alkane and aromatic compound degradation (Hx and PAHs, respectively) was obtained from the eight wells corresponding to the highest positive dilution of plates of OR and OS samples subjected to MPN analysis. Cells were harvested from wells, lysed with sodium dodecyl sulfate (10%), lysozyme, and proteinase K; treated with 10% cetyltrimethylammonium bromide; and freeze-thawed three times with liquid nitrogen and a 65°C bath. The extracted DNA was purified by phenol-chloroform-isoamyl alcohol extraction as previously described (10, 67).

DGGE. Genomic DNA from OR, OS, NOS, MPN analysis microtiter plates, and hydrocarbon-degrading strains were subjected to DGGE analysis. 16S rRNA gene hypervariable regions V3 to V5 were amplified with primers 16F341-GC and 16R907 (73). Primer F341-GC included a GC clamp at the 5′ end (5′-CGCCCGC CGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′). In this case, PCRs were performed in a volume of 50 μl containing 1.25 U of *Taq* (TaKaRa ExTaq Hot Start Version; TaKaRa Bio Inc., Otsu, Shiga, Japan), 1× ExTaq buffer (2 mM $MgCl_2$), 200 μM each deoxynucleoside triphosphate, 0.5 μM primers, and 100 ng of template DNA. After 9 min of initial denaturation at 95°C, a touchdown thermal profile protocol was carried out and the annealing temperature was decreased by 1°C per cycle from 65 to 55°C, followed by 20 additional cycles of 1 min of denaturation at 94°C, 1 min of primer annealing at 55°C, and 1.5 min of primer extension at 72°C and then a final 10 min of primer extension at 72°C.

Approximately 800 ng of purified PCR product was loaded onto a 6% (wt/vol) polyacrylamide gel that was 0.75 mm thick with denaturing gradients and denaturant concentrations that ranged from 40 to 75% (100% denaturant contained 7 M urea and 40% formamide). DGGE was performed in 1× TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 8.4) with a DGGE-2001 system (CBS Scientific Company, Del Mar, CA) at 100 V and 60°C for 16 h. DGGE gels were stained with 1× TAE buffer containing Sybr gold (Molecular Probes, Inc., Eugene, OR). Predominant DGGE bands were excised with a sterile razor blade, suspended in 50 μl sterilized MilliQ water, stored at 4°C overnight, reamplified by PCR with primers F341 and R907, and cloned with a TOPO TA cloning kit (Invitrogen) as described below.

Analysis of DGGE images. Bacterial diversity analysis and correlation principal-component analysis (PCA) of band types were performed, and the relative peak areas were calculated as previously described (62) for the different DGGE profiles (i.e., OR, OS, NOS, OR-Hx, OR-PAH, OS-Hx and OS-PAH [Fig. 4]) to consider possible shifts in the composition of the microbial populations. A dendrogram was constructed by the nearest-neighbor cluster method with the

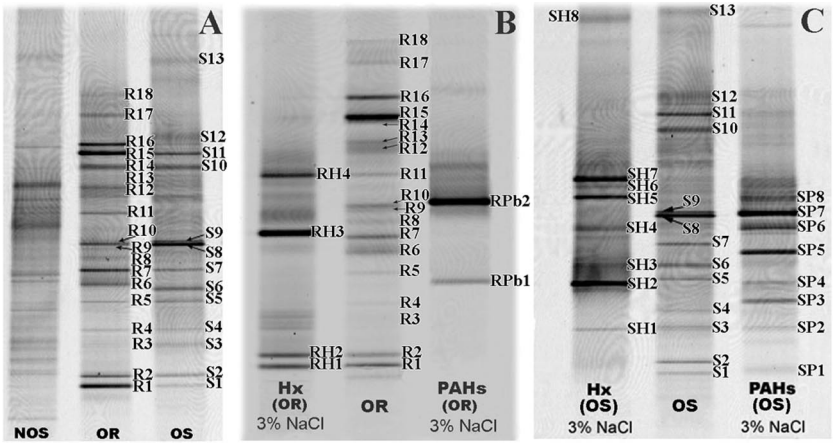

FIG. 4. DGGE profiles of PCR-amplified 16S rRNA genes of bacterial communities from oiled samples (OR and OS) compared with those of bacterial communities from NOS (A). The hydrocarbon (alkane [Hx] and aromatic compound [PAH])-degrading bacterial populations from polluted rock (B) and sand (C) are compared with their total profiles (OR, OS). R1 to R18 and S1 to S13 indicate excised and sequenced bands from the total OR and OS profiles, respectively (see Table S2 in the supplemental material). Bands of the alkane (OR-Hx and OS-Hx)- and aromatic (ORPx and OSPx)-degrading populations from rock and sand, respectively, were sequenced as well (see Table S3 in the supplemental material). Bacterial diversity (Table 3), PCA (see Fig. S4 in the supplemental material), and a neighbor-joining tree from Pearson correlation factor data (see Fig. 5) were obtained from the complete densitometric curves of the different DGGE profiles (i.e., OR, OS, NOS, OR-Hx, OR-PAH, OS-Hx, and OS-PAH) to consider possible shifts in the composition of the microbial populations.

Pearson product-moment correlation coefficients calculated from the complete densitometric curves for the fingerprints of the different bacterial communities.

16S rRNA gene clone library. Almost the complete 16S rRNA gene was amplified from OS and OR genomic DNA with primers F27 and R1492 as previously described (19, 35). The PCR mixture (25 μl) included 10 mM Tris HCl (pH 8.3), 50 mM KCl (pH 8.3), 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1.25 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA), 0.4 μM each primer, and 100 ng of DNA extracted from either OR or OS samples. The reaction mixtures were subjected to an initial denaturation and enzyme activation step (5 min at 95°C); 40 cycles of 30 s at 96°C, 30 s at 54°C, and 1.5 min at 72°C; and an extension step of 10 min at 72°C.

PCR products were ligated into the pCR2.1-TOPO vector and transformed into competent *Escherichia coli* TOP 10F′ cells by following the protocol of the manufacturer of the TOPO T/A cloning kit (Invitrogen). Restriction fragment length polymorphism analysis of the clones was performed to identify clone representatives of different enzyme restriction patterns, digesting the PCR products separately with 5 U of AluI and TaqI (Amersham Biosciences, Uppsala, Sweden) for 3 h at 37°C and 65°C, respectively.

PCR products from recombinant clones and the resulting restriction enzyme fragment patterns were separated by electrophoresis in a 1% and 3% (wt/vol) agarose gel in 1× TAE buffer, respectively, stained with ethidium bromide, and photographed under UV light with a Gel Doc XR system and Quantity One software (Bio-Rad, Hercules, CA). Clones representatives of different enzyme restriction patterns were sequenced in both directions with internal primers F341 and R907 (19).

Sequencing and phylogenetic analysis. Sequencing was accomplished with the ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (version 3.1) and an ABI PRISM 3700 automated sequencer (PE Applied Biosystems, Foster City, CA) by following the manufacturer's instructions. 16S rRNA genes were partially sequenced in both directions with primers F341 and R907 (19). Sequences were inspected, assembled, subjected to the Check Chimera program of the Ribosomal Database Project (41), and examined with the BLAST search alignment tool comparison software (BLASTN) (4) to detect the bacterial group in the GenBank database closest to each strain.

Sequences were aligned with reference sequences obtained from GenBank, and phylogenetic analyses were performed as previously described (3) to better classify the detected bacteria.

Nucleotide sequence accession numbers. The 363 nucleotide sequences identified in this study have been deposited in the GenBank database under accession numbers EU374875 to EU375237.

RESULTS

Chemical analysis. The gas chromatographic profiles of the aliphatic fractions evidenced petrogenic contamination based on the occurrence of the homologous series of C_{15} to C_{40} *n*-alkanes overlying an unresolved complex mixture of hydrocarbons (see Fig. S1 in the supplemental material). The confirmation of the presence of the *Prestige* oil was obtained by a detailed study of the fossil biomarkers, namely, steranes and triterpanes, currently used for oil spill fingerprinting (15). The diagnostic molecular parameters of the oiled samples indicated a clear correspondence to those of the fuel oil (see Fig. S2 in the supplemental material), whereas those of the control sand sample (NOS) exhibited a different pattern. The ratios of C_2 and C_3 dibenzothiophenes (D2 and D3) and phenanthrene/anthracenes (P2 and P3), proposed for differentiating sources of spilled oils in sediments (17), also supported the presence of the *Prestige* oil in the collected samples (see Fig. S2 in the supplemental material).

The occurrence of biodegradation was assessed by the depletion of certain components with respect to those more refractory, such as triterpanes (e.g., hopane), and by changes in relative distributions within isomeric series (e.g., alkyl C_1- and C_2-phenanthrenes, dibenzothiophenes). In summary, the *n*-alkanes were severely depleted in the lower fraction ($<n$-C_{20}) as a result of weathering, but the ones in the higher fraction were also, which should be attributed to biodegradation (see Fig. S3A in the supplemental material).

Enumeration of heterotrophic, alkane-degrading, and aromatic-degrading microbial populations. While the total heterotrophic bacteria in the oiled and nonoiled sands (OS and NOS) presented similar abundances (10^5 to 10^6 microorganisms per g of sample), hydrocarbon-degrading populations were 10- to 100-fold greater in the oiled sample than in NOS (Fig. 3). The alkane-related populations found in OR and

OS were also similar (around 10^4 to 10^5 microorganisms · g^{-1}), accounting for more than 50% of the heterotrophic bacteria and always greater than the aromatic one. However, aromatic-degrading bacterial counts were 10-fold higher in the polluted sand (10^3 -10^4 microorganisms · g^{-1}) than in the oiled rocks.

Isolation of culturable strains. Around 40 morphologically different strains were isolated on MA 1/5 directly from each sample (RPx and APx strains), whereas more than 20 strains were isolated in the same medium from the MPN analysis plates of populations related to *n*-hexadecane and aromatic hydrocarbon degradation (RPHx and RPPx strains from OR and APHx, APPx strains from OS; Table 1).

With selective medium for isolation of alkane degraders (BMTM agar plus *n*-hexadecane), approximately 20 additional strains were isolated directly from rock (PDRx strains) and sand (PDAx strains) samples, respectively. Finally, 15 additional strains from each sample were isolated in phenanthrene agar from an enrichment culture grown on phenanthrene (0.05%, wt/vol) at 150 rpm and 25°C for more than 2 weeks (strains PhRx and PhSx, Table 1).

All of the strains isolated from polluted sites (RP and AP) were sequenced, but in the other cases (RPH, RPP, APH, APP, PDA, PDR, PhR, and PhS) only those strains suspected of having some degrading capacity or with the same migration length as any of the OR/OS-related DGGE bands were analyzed further (Table 1).

Screening of hydrocarbon-degrading capability. Alkane-degrading activity was found in isolated strains from both of the environments studied (sand and rock), and although the percentage of degraders varied depending on the medium used, it was always dominated by *Actinobacteria* (see Table S1 in the supplemental material). In general, polluted sand samples (see Table S6 in the supplemental material) presented a much higher percentage of hydrocarbon-degrading strains than did polluted rock samples (see Table S5 in the supplemental material), even with nonselective medium (33% of AP strains compared to 2.6% of RP strains). As expected, the use of a selective medium (hydrocarbon-agar) was the best strategy to isolate alkane-degrading strains (72 and 81% of the PDR and PDA strains were alkane degraders; see Table S1 in the supplemental material) and almost the only way to isolate bacteria related to PAH degradation (PhR/PhS strains). Eleven PhR and nine PhS isolates (representing 73 and 60% of the total) from the OS and OR phenanthrene enrichments grew as pure cultures on PAHs. These strains belonged to only two species of *Pseudomonas* and *Sphingomonas* (Table 2).

DGGE profiles of the total bacterial community. Cluster analysis and PCA indicated that the bacterial communities from oiled samples (OR and OS) were quite similar, while the nonoiled control (NOS) was the most distantly related (Fig. 5; see Fig. S4 in the supplemental material). Five OR DGGE bands, R1, R2, R7, R14, and R15, were identical in sequence to OS DGGE bands S1, S2, S7, S10, and S11, respectively. Those bands were related to the genus *Rhodococcus*, uncultured *Rhodobacteraceae*, *Lutibacterium*, and *Chromatiales*, respectively. Additional bands from these and other organisms related to oil degradation, such as *Citreicella* spp. (bands R9, R10, and R11), *Sphingopyxis* spp. (bands R12 and R17), *Erythrobacter* spp. (band R16), and *Yeosuana aromativorans* (band S13), were also found in total DGGE profiles (Table 2 see Table S2 in the supplemental material).

DGGE profiles of presumably oil-degrading bacterial populations. PCA (see Fig. S4 in the supplemental material) of excised and nonexcised bands from the different DGGE profiles (Fig. 4B and C) suggested that OR community members were mainly related to alkane degradation while the oiled sand community included members related to the degradation of both fractions. However, 16S rRNA gene sequences were obtained only from the most conspicuous bands of the profiles and no sequences common to populations related to the degradation of alkanes (Hx) and aromatics (PAHs) (see Table S3 in the supplemental material) and the total community (OR, OS) could be confirmed (see Table S2 in the supplemental material). An exception was found with bands RH1 and RH2 from the presumably *n*-hexadecane-degrading population profile of the rock sample (OR-Hx). These bands were, respectively, identical to the *Rhodococcus* bands shared by the total-community profiles of the oiled rock and sand (RH1 = R1 = S1 and RH2 = R2 = S2; Table 2).

Higher diversity related to alkane and aromatic degradation was found in the sand than in the oiled rocks (Table 3). However, some common bands within the OR-Hx profiles (RH4) and OS-Hx (SH5, SH6) were found related to *Pseudoxanthomonas spadix* (99 to 100% similarity) (Table 2). Other *Xanthomonadaceae* genera related to alkane degradation in OS were close to *Dokdonella koreensis* (bands HA2/HA3) and to *Stenotrophomonas maltophilia* (band SH8). The *Alphaproteobacteria* genus *Erythrobacter* was detected as one of the most conspicuous of the OS-Hx profile (SH7).

The OR-PAH DGGE profile was composed of only two dominant bands (Fig. 4B), RPb1 and RPb2, corresponding to the genera *Tistrella* and *Sphingomonas*, whereas eight bands could be excised and sequenced from the OS-PAH DGGE profile (Fig. 4C; see Table S3 in the supplemental material). It is important to point out that band RPb2 (OR-PAHs) was identical to SP7 (OS-PAHs), being close to *Sphingomonas* spp. Another three similar sequences from sand, bands PA5, PA6, and PA8, were also related to the genus *Sphingomonas* (Table 2).

Clone libraries. As explained in the following section, to obtain an image of the most abundant genera present in each matrix community (Table 2; see Table S4 in the supplemental material), approximately 70 clones were sequenced for each sample (OR and OS).

Oiled rock (OR) sample total community. The main bacterial groups found in OR were the classes *Alphaproteobacteria* (43%; the genera *Parvibaculum* and *Lutibacterium*), *Actinobacteria* (28%; *Rhodococcus*, *Dietzia*, and *Microbacterium* spp.), and *Gammaproteobacteria* (23%; *Salinisphaera*, *Chromatiales*, and *Alcanivorax* spp.) (see Table S1 in the supplemental material). The most important genus was *Rhodococcus*, which was represented by seven different sequences accounting for 20% of the total library. Two of these sequences, with the highest frequencies, were identical to DGGE bands R1 and R2, respectively (Table 2). Phylogenetic analysis placed them close to *Rhodococcus fascians* DSM20669 (99 and 98% similarity, respectively). A minor presence of clones related to *Alcanivorax* spp. (3 out of 65) was found, and only 1 of the 65 was identical to *Alcanivorax borkumensis*. Members close to the *Chromatia-*

TABLE 2. Summary of the most interesting 16S rRNA sequences detected from DGGE bands, clones, and degrading strains from OR and OS samples[a]

Phylogenetic group[b]	Closest classified organism from GenBank database (accession no.) in phylogenetic tree[c]	DGGE band	Clone library (no. of clones/ total)		Degrading isolates[d]		Importance for bioremediation[e]	
			OR	OS	OR	OS	This study	Other study(ies) (reference[s])
Alcanivoraceae (γ)	*Alcanivorax* spp. (AM286690, DQ347532, AY258109, AY683537)		3/65	3/72		3/29°	Hx	30, 70
Chromatiales (γ)	Uncultured gammaproteobacterium (DQ870518)	R15=S11	4/65	1/72				27
	Uncultured soil bacterium clone M54-Pitesti (DQ378269)	S8	1/65	4/72				
Pseudomonadaceae (γ)	*Pseudomonas* spp. (AY691188, AJ312176)				2/11*	6/9*	Phe	18, 45, etc.
Salinisphaeraceae (γ)	*Salinisphaera* sp. strain ARD M17 (AB167073)		6/65					
Xanthomonadaceae (γ)	*Dokdonella koreensis* DS-140 (AY987369)	SH2/SH3					Hx (?)	
	Pseudoxanthomonas spadix IMMIBAFH-5 (AM418384)	RH4/SH5/SH6					Hx (?)	13, 72
	Stenotrophomonas sp. strain KL1A1 (DQ208664)	SH8					Hx (?)	31, 71
Erythrobacteraceae (α)	*Erythrobacter* sp. strain JL893 (DQ985055)	R16=SH7	1/65				Hx (?)	27, 40, 44, 50
Phyllobacteriaceae (α)	*Parvibaculum lavamentivorans* (AY387398)	R6/SH1	5/65	1/72				52, 53
Rhodobacteraceae (α)	Uncultured *Rhodobacteraceae* bacterium (DQ870525)	R7=S7	3/65					27
	Citreicella sp. strain 2-2A (AB266065)	R9/R10/R11	1/65				Phe (?)	K. Watanabe et al., unp.
Rhodospirillaceae (α)	*Thalassospira* sp. strain DBT-2 (DQ659435)	RH3		1/72				B. Wang et al., unp.
	Tistrella mobilis (AB071665)	RPb1					Phe (?)	44
Sphingomonadaceae (α)	*Lutibacterium anuloederans* (AY026916)	R13/R14=S10/S6	6/65	5/72				14
	Novosphingobium spp. (AY690709, AJ416411)		3/65	1/72				29
	Sphingomonas spp. (AY646154, AJ717392, AY690679, AB099636, AF282616)	SP5/SP6/SP8 RPb2=SP7	4/65	1/72	9/11*	3/9*	Phe	Y. Ahn, unp,; 22, 56
	Sphingopyxis sp. strain FR1093 (DQ781321)	R12/R17						
Flavobacteriaceae (B)	*Yeosuana aromativorans* (AY682382)	S13		1/72				34
Dietziaceae (A)	*Dietzia* spp. (AB159036, X79290)		3/65		3/26°		Hx	11, 49, 74
Gordoniaceae (A)	*Gordonia polyisoprenivorans* (DQ154925)					3/29°	Hx	11, 39
Microbacteriaceae (A)	*Microbacterium* spp. VKM Ac-2048 (AB042083)		2/65					24, 51
Mycobacteriaceae (A)	*Mycobacterium* spp. (AY255478, AJ276274, AY235429, DQ372728)			5/72				8, 25, 32, 66
Nocardiaceae (A)	*Rhodococcus* sp. strain 5/1 (AF181689)	R1=S1=RH1/R3/R5	11/65	5/72	15/26°	14/29°	Hx	9, 26, 46, 55
	Rhodococcus sp. strain MBIC01430 (AB088667)	R2=S2=RH2/S3	2/65	1/72	8/26°	8/29°	Hx	26, 46, 55
	Rhodococcus opacus ML0004 (DQ474758)					1/29°	Hx	26, 46, 55
Williamsiaceae (A)	*Williamsia* sp. strain MT8 (AY894336)	R4		2/72				

[a] Designations and accession numbers for sequences and levels of similarity to related organisms are shown. Information from this study comes from isolated strains that gave positive results in alkane or aromatic (Hx and Phe, respectively) degradation tests. Although it is not totally appropriate to draw conclusions about physiological features from molecular data, references to other studies reporting any relationship of the sequence detected to biodegradation (degrading capacity, detection in hydrocarbon-polluted samples. . .) are given.

[b] γ, α, and δ represent gamma-, alpha-, and deltaproteobacteria, respectively. B, *Bacteroidetes*; A, *Actinobacteria*; F, *Firmicutes*; P, *Planctomycetes*; C, *Chloroflexi*.

[c] Sequences were matched with the closest relative from the GenBank database after a BLAST search and phylogenetic analysis.

[d] Proportion of each degrading isolated species relative to the total number of isolates able to degrade the same fraction in each polluted matrix. Symbols: =, identical sequence; /, similar sequence; °, alkane-degrading isolates; *, aromatic-degrading isolates.

[e] Compilation of the available data from this and other studies about the degrading ability related to each 16S rRNA gene sequence. ?, related to alkane or aromatic degradation but needs further study of its specific role; unp., unpublished data. Normally refers to the authors of the closest sequence in GenBank (accession number).

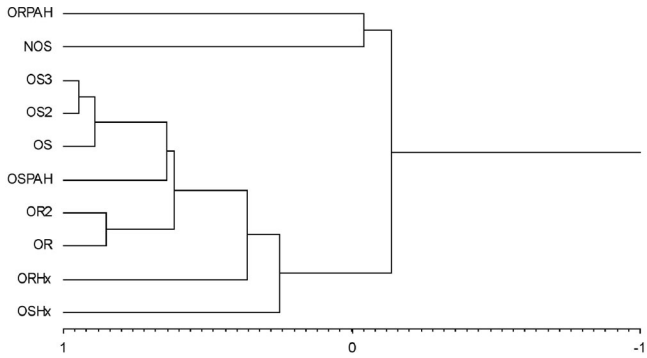

FIG. 5. Cluster analysis from a similarity matrix generated from DGGE profiles (Fig. 4) according to the Pearson product moment and the unweighted-pair group method using average linkages. The DGGE profile of OS samples was close to that of hydrocarbon degraders, whereas the OR DGGE profile was more similar to that of alkane degraders than to that of PAH degraders. NOS, nonoiled sand; OS plus a number, oiled sand replicates; OR plus a number, oiled rock replicates. OR-Hx, OR-PAH, OS-Hx, and OS-PAH, total DNA from bacteria growing at the highest dilutions in hexadecane (Hx) and aromatic (PAH) MPN analyses, respectively.

les group, the genera *Parvibaculum* and *Lutibacterium*, detected in the DGGE profiles (see Tables S2 and S3 in the supplemental material) were also found in the clone libraries (7 to 12% of the clones; Table 2; see Table S4 in the supplemental material). The genus *Salinisphaera* (97 to 98% similarity), although not detected by DGGE, constituted a high percentage of the library (9%). The *Bacteroidetes* group (6%) was also found and was represented by four different genera (see Table S4 in the supplemental material).

Oiled sand (OS) sample total community. A higher species richness was found in OS than in OR samples (see Table S4 in the supplemental material). The main bacterial groups were the class *Alphaproteobacteria* (38% of the clones, including 20 different genera), the class *Actinobacteria* (30% of the clones, including the genera *Rhodococcus* [8%] and *Mycobacterium* [7%]), and the class *Gammaproteobacteria* (19% of the clones). In contrast to OR samples, OS samples contained other minor representatives such as members of the *Deltaproteobacteria*, *Planctomycetes*, and *Chloroflexi* groups. The *Bacteroidetes* group also presented a notable number of species (see Table S4 in the supplemental material). *Lutibacterium anuloederans* (96 to 99% similarity) clones, similar to bands S10 and S6 (Fig. 4A), accounted for 6% of the clones. Most of the clones related to *Rhodococcus* were again identical to DGGE band S1 (99% similarity to *R. fascians* DSM20669). Most of the members of the class *Gammaproteobacteria* were close to sequences of uncultured *Chromatiales* and identical to bands S8 (6%) and S9 (3%), while *Alcanivorax* was detected again in low abundance (4%) and only 1 clone out of 72 was identical to *A. borkumensis*.

Bacterial isolates. Alkane-degrading strains isolated from the OR sample belonged exclusively to the genera *Rhodococcus* and *Dietzia* of the class *Actinobacteria* (see Table S5 in the supplemental material). In fact, 26 out of the 32 positive alkane-degrading strains matched exactly either the R1 = S1 = RH1 or the R2 = S2 = RH2 band sequences from DGGE belonging to the genus *Rhodococcus* (Fig. 6A), whereas 3 out of 32 were related to the genus *Dietzia*. *Rhodococcus* type 1 and 2 strains were, respectively, identical to *Rhodococcus* sp. strain 5/1 (accession no. AF181689) and 99% similar to *Rhodococcus* sp. strain MBIC01430 (accession no. AB088667) (Table 2). *Dietzia*-related strains (e.g., PDR4 or PDR22), close to *D. maris* (99 to 100% similarity) and *D. psychralkaliphila* (99 to 100% similarity), migrated close to *Rhodococcus* bands (Fig. 6A). Both belong to the class *Actinobacteria*, which is characterized by a high G+C content and thus stability in its 16S rRNA gene sequences, which migrated longer in the DGGE gel. The different strains of *Rhodococcus* seemed to grow very close in hexadecane culture, being indistinguishable and difficult to isolate. DGGE helped to detect those nonpure cultures such as PDR23 which were a mixture of the two *Rhodococcus* strains, types 1 and 2 (Fig. 6A). Strains were separated afterward with marine agar, where the different strains developed different colors and morphologies. Although isolates mainly from the enrichment cultures in hexadecane (PDA) also confirmed the dominance of *Rhodococcus* (16 out of 26; 80% similarity), a higher number of additional species related to alkane degradation (e.g., *Gordonia*, *Erythrobacter*, *Stenotrophomonas*, and *Alcanivorax* spp.; Fig. 6B; see Table S6 in the supplemental material) could be isolated than from OR (Fig. 6A; see Table S5 in the supplemental material). Even though isolates of both *Erythrobacter* (99% similar to bands SH7 and R16 from the OS-Hx and OR DGGE profiles, respectively) and *Stenotrophomonas* (identical to band SH8) were detected in the population related to alkane degradation, no degrading ability could be confirmed, in contrast to *Dietzia*, *Rhodococcus*, *Gordonia*, and *Alcanivorax* isolates, which were able to grow on hexadecane as the only source of C and energy (Table 2).

All isolates related to PAH degradation were obtained from phenanthrene enrichments (PhR and PhS strains), like *Sphingomonas*, *Pseudomonas stutzeri*, and *Tistrella mobilis* (Fig. 6C and Table 2; see Table S1 in the supplemental material). One exception occurred with *Citreicella* strain RP3, which could only be isolated with one-fifth-strength marine agar directly from fuel oil attached to rocks (OR). Aromatic-degrading ability could be confirmed only in species of two genera, *Sphingomonas* and *Pseudomonas*. PAH-degrading strains of *Sphingomonas* were close to DGGE bands of OS-PAHs (SP5-SP8),

TABLE 3. Shannon-Weaver diversity indexes calculated for DGGE profiles and numbers of DGGE bands detected[a]

Sample	Shannon-Weaver diversity index	No. of DGGE bands (richness)
OR	1.24	20
OR2	1.06	18
OS	1.26	22
OS2	1.33	22
OS3	1.25	24
NOS	1.38	27
OR-Hx	0.91	11
OS-Hx	1.03	13
OR-PAH	0.46	6
OS-PAH	1.07	16

[a] From Fig. 4 and 6. NOS, nonoiled sand; OS plus a number, oiled sand replicate; OR2, oiled rock replicate; OR-Hx, OR-PAH, OS-Hx, and OS-PAH, total DNA from bacteria growing at the highest dilutions in hexadecane (Hx) and aromatic (PAH) MPN analyses, respectively.

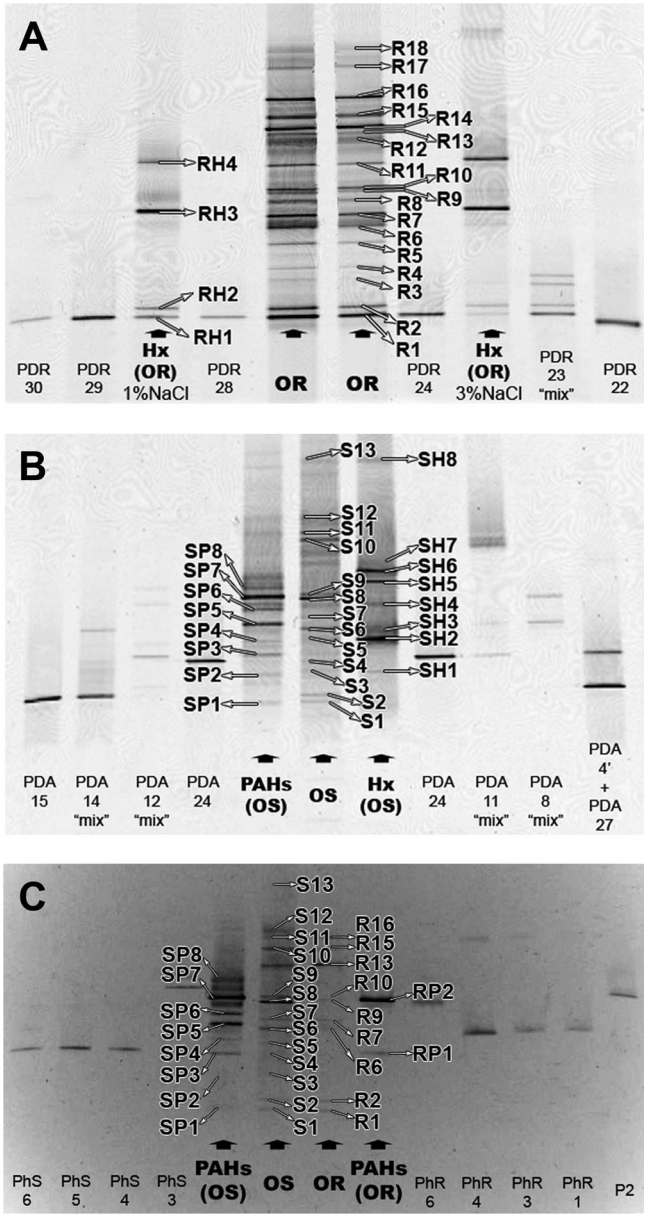

FIG. 6. DGGE screening of isolated alkane-degrading strains from rocks (A) and sand (B) and isolates related to PAH degradation from both matrices (C). DGGE patterns of total communities (OR and OS) and their respective hydrocarbon (Hx and PAH)-degrading populations were used as references to detect those strains playing roles in the biodegradation of the *Prestige* crude oil in the environment (e.g., hexadecane-degrading strain PDR24, identical to bands R1 and RH1, indicates the presence of *Rhodococcus* and its role in the in situ degradation of the alkane fraction of the *Prestige* fuel). The OR, OS, OR-Hx, OS-Hx, OR-PAH, and OS-PAH profiles, marked with arrows and bold letters, were identical to those described in Fig. 4. Some of the sequenced bands are referenced again in the present figure to assist in the detection of strains. The term "mix" indicate those isolates which were mixtures of more than one strain (e.g., PDR23 was composed of two strains of *Rhodococcus*, where the upper two bands were a heteroduplex of the two *Rhodococcus* 16S rRNA gene sequences).

and one of them was identical to bands RPb2 and SP7 (Table 2). *Tistrella* isolates had a sequence identical to DGGE band RPb1 (OR-PAHs) and therefore are related to the degradation of aromatics, although no ability could be confirmed. Something similar occurred with *Citreicella* strain RP3, which had a 16S rRNA gene sequence close to DGGE bands R9 and R10 and identical to band R11 and clone Rc10 (accession no. EU375056) from OR samples (Table 2). The strain was close (99 to 100% similarity) to *Citreicella* sp. strain 2-2A (accession no. AB266065), although no degrading activity could be observed in our strain with the methodology used.

DISCUSSION

Impact of fuel on microbial populations. The *Prestige* oil spill did not affect bacterial abundances in the areas studied but induced deep changes in the trophic structure of bacterial communities. A similar situation was described after the *Nakhodka* oil spill, with a composition similar to that of the *Prestige* fuel, in the marine communities of the Japan Sea (31, 43). Although the communities were qualitatively different from those in NOS, those affected by the oil spill still conserved high species richness and diversity. These results agree with previous observations that community diversity was dramatically reduced just after the pollution event and progressively recovered to preoiling levels but with a different structure dominated by hydrocarbonoclastic bacteria (24, 50). In the present study, we observed that although rocks and sand are quite different substrates, community compositions were quite similar, suggesting that fuel oil drives the structure of the communities affected. However, the higher species richness and diversity of OS communities detected by culture-dependent and -independent methods suggests that the environmental conditions on the OR surface, subject to daily contrasting temperatures and dryness, may require a more specialized microbial population to survive under such restricting conditions compared to those that exist in sand, where a higher number of different bacteria can grow.

Predominance of taxonomic groups and microbial diversity. Most previous studies have focused on the short-term effects of crude oil or its components on marine bacterial communities, which usually became dominated by *Gammaproteobacteria* (1, 24, 50) just after an oil spill. In artificially oiled environments amended with nutrients, biodegradation rates were promoted and the first fast petroleum degradation processes were carried out by communities dominated by *Gammaproteobacteria* (e.g., *Alcanivorax*, *Cycloclasticus*, *Thalassolituus*…), which were rapidly replaced by *Alphaproteobacteria* in less than a month (50). In the present work, as previously done after the *Nakhodka* oil spill in the Japan Sea (31), we focused on the analysis of communities affected for a long time by heavy fuel oil. In the affected coasts of the Japan Sea, where natural attenuation proceeded slowly, probably due to the small amount of nutrients present (total N, ~0.1 mg liter^{-1}), bacterial communities from oil paste were still dominated by *Gamma-* and *Alphaproteobacteria* (gram-negative) more than 12 months after the oil spill (31), indicating that oil from the *Nakhodka* was still rich in those more biodegradable fractions due to slow degradation processes. However, *Alphaproteobacteria* and gram-positive *Actinobacteria* dominated our oiled samples after the same time. Gram-positive bacteria do not respond to high hydrocarbon inputs (42) and are never dominant just after an oil spill, being detected in nonpolluted areas (33, 40) or in long-weathered oil-polluted environments (48). The differences observed

between the molecular marker ratios of the original fuel oil and those of the oiled samples (see Fig. S2 in the supplemental material) are also consistent with the trends that follow biodegradation (28). The aromatic fraction exhibited a predominance of alkylnaphthalenes in the original oil that were almost lost in the collected samples mainly by water washing and evaporation (see Fig. S3B in the supplemental material). However, microbial degradation was observed as a severe depletion of the *n*-alkane fraction, even the higher fraction (see Fig. S3A in the supplemental material), and the relative reduction of isomers with β substituents such as the 2- and 3-methylphenanthrenes and dibenzothiophenes within their respective series (28). This enrichment in more recalcitrant fractions of the fuel might explain the dominance of gram-positive bacteria previously hypothesized to have roles in the degradation of such less biodegradable hydrocarbon classes (48).

Since the oil from the *Nakhodka* had a composition similar to that from the *Prestige* (heavy fuel) and the time of sampling was the same, it seems likely that the higher level of nutrients (0.20 to 0.25 mg liter^{-1} total N, of which 0.15 mg ml^{-1} was nitrate [5]) supplied to the littoral of the Costa da Morte (northwestern Spain) by the northwestern Africa upwelling system (20) is responsible for the observed differences in the biodegradation rate (68) and community composition.

Alcanivorax (70) dominates oil-degrading communities when nutrients are supplied, but with normal levels of nutrients more diverse communities can exist (30, 50). In a recent study, we reported the presence of *A. borkumensis* in high numbers just after the *Prestige* oil spill in sediments of the Ría de Vigo (3), where the alkane fraction was still abundant and higher nutrient levels (0.6 mg liter^{-1} total N) than at the Costa da Morte (0.20 to 0.25 mg liter^{-1} total N) existed (5). In this sense, members of the well-described hydrocarbonoclastic genus *Alcanivorax* were still present in OR and OS but in very low numbers, as occurred in marine environments affected for long times by heavy fuels (31, 48).

DGGE profile differences among the different trophic populations detected and the reduction in the number of bands with respect to the total profiles (Table 3) suggested an important specialization of species roles in the process of fuel biodegradation in both matrices.

Population related to alkane degradation. Culture-independent and -dependent analyses showed that *Actinobacteria*, mainly *Rhodococcus* species, was the key alkane-degrading group of bacteria. *Rhodococcus* has been associated with the degradation of *n*-alkanes up to C_{36} (65) and branched alkanes (64), which are particularly abundant in the *Prestige* fuel (16). It is well known that *Rhodococcus* is a genus with remarkable metabolic diversity (36) and is able to produce biosurfactants which can enhance not only the bioavailability of fuel components but also the growth of other degrading bacteria (26, 47). *Dietzia* and *Microbacterium* species, detected exclusively in the OR clone library, have been, respectively, described as degraders of alkanes, including branched alkanes (49, 74), or related to oil degradation in hydrocarbon-polluted sites (24, 51). Since *Dietzia*, *Microbacterium*, and *Rhodococcus* belong to the class *Actinobacteria*, some common characteristic might explain the dominance of this group on OR. In this sense, an interesting study which compared the different uptakes of hydrocarbons by two *Pseudomonas* and *Rhodococcus* strains (59) clearly showed how the hydrophobic surface developed by the latter allowed the growth of *Rhodococcus* attached to the oil surface increasing its degrading capacity. This capacity might explain the relative major presence of *Rhodococcus* on OR compared to OS since this ability could represent an important advantage for survival in such a harsh environment.

Several members of the family *Xanthomonadaceae* that were detected in this study were associated with the degradation of alkanes since they were detected in Hx and PAH DGGE profiles (Fig. 4B and C and Table 2). In fact, with the exception of *D. koreensis*, which was not previously related to either alkane degradation or oil-polluted sites, the other species (*P. spadix* and *S. maltophilia*) were previously associated with oil degradation and surfactant production (13, 72). The alphaproteobacterium genus *Erythrobacter*, commonly encountered after first fast degradation processes (40, 50), was detected as well as part of the population related to alkane degradation (OS-Hx). Since no ability to degrade hydrocarbons could be confirmed for any of these strains, they might play secondary roles in the degradation of this fraction in collaboration with *Actinobacteria*, mainly in OS, where a higher diversity existed (Table 3).

Populations related to aromatic degradation. The metabolism of PAHs is a more complex process than the metabolism of the aliphatic fraction, where the initial bacterial dioxygenases from PAH metabolism exhibited a lower substrate specificity. Frequently, the resulting oxidized PAHs require the intervention of another bacterial strain, which plays an important role in degradation but cannot be detected as an aromatic hydrocarbon degrader. In this sense, we described in a previous work how the bacterial metabolism of fluorene needed the coculture of two strains, of which only one was able to degrade the aromatic while the other eliminated secondary metabolites produced by the former (12). Probably this laboratory model reproduces a very frequent metabolic cooperation among different strains in the bacterial metabolism of PAHs in situ.

Strains related to *Sphingomonas* were isolated as phenanthrene-degrading strains in both matrices (Table 2). The aromatic-degrading *Sphingomonas* isolates in this study were quite different from the single clone (Sc29) detected, and thus no significance in the in situ degradation of the fuel mixture can be attributed to these strains. However, two DGGE bands (R12 and R17) and 4.5% of the OR library were related to *Sphingopyxis* and *Novosphingobium*, respectively (Table 2), two genera formerly considered to be *Sphingomonas* (57). Moreover, oil paste from a beach affected by the *Nakhodka* oil spill presented sequences related to *Sphingomonas subarctica* (100% similarity) which were proposed to play roles in PAH degradation (31). Interestingly, other members of this family could play a central role in the degradation of the aromatic fraction of the *Prestige* fuel in both matrices, such as *L. anuloederans* (95 to 100% similarity; two DGGE bands and around 7% of the clones of each sample; Table 2). Although we could not isolate any strain of this species, *L. anuloederans* was described as a two- and three-ring PAH-degrading bacterium which had a higher efficiency in the uptake of aromatics than *Cycloclasticus* species do (14). Similar results have been recently observed in a complete study performed in the Thames estuary (United Kingdom), where *Cycloclasticus* seemed to dominate seawater microcosms spiked with single

PAHs except those containing fluorene, where a sequence close to *L. anuloederans* was found (44).

Several clones close to different species of *Mycobacterium* spp. were detected in OS. All of the clones were different from each other, but some were close to the species *Mycobacterium frederiksbergense* (98 to 100% similarity), which has previously been reported to mineralize the PAHs phenanthrene, fluoranthene, and pyrene (66). *Mycobacterium* species are specialized in the degradation of adsorbed PAHs in soils (8). However, the most frequently used PAH-degrading bacterial isolation methodology, including the one we used, is conducted with liquid medium with agitation (7), so those strongly adhering bacteria may tend to escape from conventional isolation techniques (8, 60), as occurred in the present study. Fortunately, other research groups could obtain aromatic-degrading isolates of *Mycobacterium* spp. from pyrene enrichments of *Prestige* oil-polluted samples which were really close to OS (M. Grifoll, personal communication; http://otvm.uvigo.es/vertimar2007/comunicaciones/VEM2004-08556_ortega.doc).

Cycloclasticus has been proposed as the main PAH degrader in many previous studies, including some done after the *Nakhodka* oil spill (43, 44). However, those studies analyzed communities from seawater samples just after the oil spill at first fast degradation processes when this and other *Gammaproteobacteria*, like *Alcanivorax*, dominated the community, while the oiled matrices under study had already suffered from weathering and biodegradation processes at the time of sampling. The ability of *L. anuloederans* and *Mycobacterium* spp. to degrade fluorene and pyrene, which are considered especially recalcitrant fuel components (63), might explain the high abundance of this bacteria in heavy fuel devoid of the most easily biodegradable fractions.

T. mobilis was previously detected in seawater microcosms spiked with PAH mixtures. In that case, it was hypothesized that this species could have a secondary role in the degradation of catabolic intermediates of aromatic compounds, owing to their appearance only after 6 or 9 weeks of incubation (44). In the present work, isolates of this strain could not grow as pure isolates on PAHs with the methodology used. However, in combination with the degrading *Sphingomonas* strain isolated, a big growth of *Tistrella* could be observed. Although further studies of the specific implication of *T. mobilis* in the aromatic degradation process are needed, our observations suggest the existence of a metabolic collaboration between them, where *T. mobilis* probably grows on second metabolites derived from the phenanthrene degradation carried out by *Sphingomonas*.

Strain RP3, detected by culture-dependent and -independent methods on OR, was close (99 to 100% similarity) to *Citreicella* sp. strain 2-2A (accession no. AB266065). This strain, first isolated from seawater as a PAH-degrading bacterium by Y. Kodama and K. Watanabe in 2006 (unpublished results), was detected with the same sequence as an "uncultured *Roseobacter* sp. (DQ870519)" in supralittoral rocks affected by the *Prestige* oil spill more than 400 km from our sampling site (27). This bacterium might need a cofactor available in the bacterial community of the oiled cobblestones to develop its degrading capacity since no growth was observed in the aromatic degradation test of RP3 in mineral medium with phenanthrene.

Ubiquity of bacterial species. Sequences close to *Rhodococcus*, *Chromatiales*, *Rhodobacteraceae*, *Roseobacter* (*Citreicella*), and *Erythrobacter* detected in both of the samples under study (OR and OS) were, respectively, identical to the sequences with accession numbers DQ870544, DQ870518, DQ870525, DQ870519, and DQ870538 retrieved from another cobblestone beach affected by the *Prestige* spill (27). In addition, several sequences found in our clone libraries showed at least 99% similarity to other DGGE bands detected in that study. What is more interesting is that DGGE profiles from that study became more similar to those of our OR and OS samples at advanced stages of the degradation process (27), which agrees with our hypothesis. Although samples were taken from rock surfaces similar to our OR, the beach was more than 400 km from our sampling point. Therefore, it seems that conclusions derived from the present work can be applied to other parts of the Spanish coast affected by the *Prestige* oil spill.

Bioremediation amendments. Mycolic acids, very-long-chain (C_{30} to C_{90}) α-alkyl, β-hydroxy fatty acids, are major and specific constituents of a distinct group of gram-positive bacteria, classified in the suborder *Corynebacterineae*, which includes genera detected in the present study such as *Mycobacterium*, *Williamsia*, *Gordonia*, *Dietzia*, and *Rhodococcus*. As opposed to gram-negative bacteria, such *Pseudomonas* or *Alcanivorax*, that dominate fast petroleum degradation processes at first (30), members of this group are never dominant at such stages (42, 48), being detected with higher frequency in resource-limited environments, where they could play a key role in the in situ degradation of more recalcitrant components a long time after an oil spill (48). Unusually, these gram-positive bacteria contain an outer permeability barrier that may explain both the limited permeability of their cell walls and their general nonsusceptibility to toxic agents (21), which has been related to an enhanced biodegradation capacity (37, 38). Therefore, the addition of mycolic acids to bioremediation amendments applied to coasts with ecological features close to those of the affected Spanish areas and affected for a long time by a contaminant similar to the *Prestige* fuel, might be a good strategy to enhance in situ degradation.

Conclusions. This study shows that supralittoral areas with favorable environmental conditions and polluted with heavy fuels are likely to be dominated, after some months of weathering and biodegradation processes, by *Actinobacteria* (mainly of the suborder *Corynebacterineae*) since this group, characterized by long-term survival in the environment even under dry, resource-limited conditions, might degrade the more recalcitrant fractions of the remaining fuel. Since the use of already acclimated indigenous microorganisms is always preferable to the use of externally inoculated degraders, the addition of mycolic acids to favor the activity of autochthonous *Corynebacterineae* is proposed for bioremediation amendments applied at later stages of bioremediation.

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Salgado for the interesting discussion of nutrient levels at the Costa da Morte.

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