Bacterial Community Dynamics and Polycyclic Aromatic Hydrocarbon Degradation during Bioremediation of Heavily Creosote-Contaminated Soil

Marc Viñas,1 Jordi Sabaté,1 María José Espuny,2 and Anna M. Solanas1*

Department of Microbiology, University of Barcelona, Diagonal 645, E-08028 Barcelona, Spain,1 and Department of Microbiology and Parasitology, University of Barcelona, Joan XXIII, s/n, E-08028 Barcelona, Spain

Received 27 December 2004/Accepted 19 July 2005

Bacterial community dynamics and biodegradation processes were examined in a highly creosote-contaminated soil undergoing a range of laboratory-based bioremediation treatments. The dynamics of the eubacterial community, the number of heterotrophs and polycyclic aromatic hydrocarbon (PAH) degraders, and the total petroleum hydrocarbon (TPH) and PAH concentrations were monitored during the bioremediation process. TPH and PAHs were significantly degraded in all treatments (72 to 79% and 83 to 87%, respectively), and the biodegradation values were higher when nutrients were not added, especially for benzo(a)anthracene and chrysene. The moisture content and aeration were determined to be the key factors associated with PAH bioremediation. Neither biosurfactant addition, bioaugmentation, nor ferric octate addition led to differences in PAH or TPH biodegradation compared to biodegradation with nutrient treatment. All treatments resulted in a high first-order degradation rate during the first 45 days, which was markedly reduced after 90 days. A sharp increase in the size of the heterotrophic and PAH-degrading microbial populations was observed, which coincided with the highest rates of TPH and PAH biodegradation. At the end of the incubation period, PAH degraders were more prevalent in samples to which nutrients had not been added. Denaturing gradient gel electrophoresis analysis and principal-component analysis confirmed that there was a remarkable shift in the composition of the bacterial community due to both the biodegradation process and the addition of nutrients. At early stages of biodegradation, the α-Proteobacteria group (genera Sphingomonas and Acospirillum) was the dominant group in all treatments. At later stages, the γ-Proteobacteria group (genus Xanthomonas), the α-Proteobacteria group (genus Sphingomonas), and the Cytophaga-Flexibacter-Bacteroides group (Bacteroidetes) were the dominant groups in the nonnutrient treatment, while the γ-Proteobacteria group (genus Xanthomonas), the β-Proteobacteria group (genera Alcaligenes and Achromobacter), and the α-Proteobacteria group (genus Sphingomonas) were the dominant groups in the nutrient treatment. This study shows that specific bacterial phylotypes are associated both with different phases of PAH degradation and with nutrient addition in a preadapted PAH-contaminated soil. Our findings also suggest that there are complex interactions between bacterial species and medium conditions that influence the biodegradation capacity of the microbial communities involved in bioremediation processes.

Polycyclic aromatic hydrocarbons (PAHs) are a class of fused-ring aromatic compounds that are ubiquitous environmental pollutants (13). Microorganisms play an important role in the degradation of PAHs in terrestrial and aquatic ecosystems, and microbial degradation is the main process in natural decontamination. Enhancement of this phenomenon is the basis of bioremediation technologies (2).

Microbial degradation of PAHs in soil is restricted by various factors that often result in a lower-than-expected bioremediation efficiency. One of these factors is the low bioavailability of the compounds. In a recent study (1) we described significant enhancement of the biodegradation of high-molecular-weight PAHs and alkylated derivatives of these compounds from Casablanca crude oil caused by a microbial consortium in the presence of a biosurfactant produced by Pseudomonas aeruginosa strain AT10. Another important factor in bioremediation of contaminated soils is the availability of nitrogen and phosphorus, which allows the necessary increase in the size of the hydrocarbon-degrading microbial populations.

Taking into account the fact that each contaminated site can respond in a different way to distinct parameters that affect microbial biodegradation, laboratory-scale bioremediation protocols have been developed in order to determine the effects of different conditions (30). In such feasibility studies, addition of nutrients, biosurfactants, exogenous inocula, or other additives can be assayed. Nevertheless, in almost all of these studies monitoring of the process is based on chemical analysis of contaminants. A better understanding of the diversity of the microbial communities inhabiting PAH-contaminated soils and their response to different biostimulation or bioaugmentation strategies could provide clues about the type of bacteria that are able to adapt to and exploit such habitats.

It is well known that the majority of microbes in environmental samples cannot be cultured at present in laboratory media, which are biased for the growth of specific microorganisms (5, 34). In light of this, molecular biological techniques offer new opportunities. For example, denaturing gradient gel electrophoresis (DGGE) allows us to directly determine the presence and relative levels of different 16S rRNA gene am-
TABLE 1. Soil treatments

<table>
<thead>
<tr>
<th>Code</th>
<th>Treatment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M</td>
<td>Untreated “dry” soil</td>
<td>Soil with 6,153% water content corresponding to 5.8% WHC</td>
</tr>
<tr>
<td>2M</td>
<td>Basic</td>
<td>Aerated soil at 40% WHC</td>
</tr>
<tr>
<td>3M</td>
<td>Autoclaved soil</td>
<td>Autoclaved three times at 121°C for 21 min</td>
</tr>
<tr>
<td>4M</td>
<td>Nutrientb</td>
<td>Aerated soil at 40% WHC; KNO3 and KH2PO4 added to give a final concentration equivalent to a C/N/P molar ratio of 300:10:1 from the total organic carbon</td>
</tr>
<tr>
<td>5M</td>
<td>Nutrient + biosurfactantb</td>
<td>Aerated soil at 40% WHC; nutrients; biosurfactant MAT10 added twice (days 0 and 130) at a final water interstitial concentration of 150 mg liter−1 at each addition</td>
</tr>
<tr>
<td>6M</td>
<td>Nutrient + inoculumb,c</td>
<td>Aerated soil at 40% WHC; nutrients; polycyclic aromatic hydrocarbon-degrading consortium AM added five times (days 14, 26, 57, 120, and 165) so that there were 106 microorganisms g of soil−1</td>
</tr>
<tr>
<td>7M</td>
<td>Nutrient + iron octoateb</td>
<td>Aerated soil at 40% WHC; nutrients; ferric ion added as ferrie octoate twice (days 0 and 145) as an oleophlic source of iron at a final interstitial water concentration of 250 μM</td>
</tr>
</tbody>
</table>

a All treatments were carried out in triplicate in 1-liter sterile glass jars containing 300 g of sieved soil (<6 mm) and were aerated and moisture corrected by mixing twice a week.

b Nutrient amendment was carried out as follows: one-third of the total nutrient addition was performed at time zero, and the remaining two-thirds was added on days 45 and 90.

c Cells from consortium AM on day 6 of incubation were grown overnight in Erlenmeyer flasks containing 500 ml of tryptone soy broth to obtain an optical density at 600 nm of 0.8 (5 × 109 total cells). Then the cells were harvested and washed in 0.85 M NaCl. The pellet was resuspended in 0.85 M NaCl and added to correct the water content in treatment 6M (approximately 30 ml).

d See reference 1 for a description of biosurfactant MAT10.

e See reference 36 for a description of consortium AM.

f See reference 33.

g See reference 36.

The soil moisture content, water-holding capacity (WHC), electrical conductivity, total organic carbon content, total nitrogen content, and pH were determined as described previously (30).

Seven different treatments in microcosms experiments designated 1M to 7M were used in the study, as shown in Table 1. To determine the best soil water content for use in the microcosm experiments, five different water contents (5%, 10%, 40%, 60%, and 75% WHC) and autoclaved soil as an abiotic control were used in the study, as shown in Table 1. To determine the best soil water content for use in the microcosm experiments, five different water contents (5%, 10%, 40%, 60%, and 75% WHC) and autoclaved soil as an abiotic control were assayed for 15 days in triplicate in miniaturized microcosms with the nutrient additions described in Table 1. The best results (P < 0.05) were observed with 40% and 60% WHC (22 to 27% biodegradation of TPH), while 20% and 75% WHC only slight biodegradation was observed (14%). Untreated soil (5% WHC) did not show significant biodegradation (P > 0.05). Thus, water content was established as a key factor for biodegradation activity, and 40% WHC was defined as the optimal water content for soil microcosm experiments.

Chemical, microbial, and molecular analyses were carried out on sampling days 0, 21, 45, 90, 135, and 200. At each sampling time, 30 g of soil was extracted as a composite sample from five points in each microcosm and stored at −20°C prior to most analyses; the only exception was microbial counting, which was performed immediately after sampling.

Monitoring the concentrations of total petroleum hydrocarbon and polycyclic aromatic hydrocarbons. Samples were dried for 16 h at room temperature and sieved (<2 mm). Before extraction, an ortho-terphenyl acetone solution was added to 2 g of sieved, dried soil as a surrogate internal standard. The spiked sample was extracted five times in an ultrasonic bath (15 min for each extraction) with 10 ml of dichloromethane-acetone (1:1; vol/vol), and the extracts were combined to obtain the total organic extract. The extracts were dried over Na2SO4 and concentrated in a rotary evaporator to dryness. The TPH fraction was obtained with an alumina chromatographic column using the EPA501 method (U.S. Environmental Agency). The TPH fraction and the PAHs were analyzed by gas chromatography with flame ionization detection using a Trace 2000 gas chromatograph (Thermo Quest, Milan, Italy) as described previously (1). Before this, a set of samples was analyzed by gas chromatography with a mass detector to verify the purity of the peaks analyzed by gas chromatography with flame ionization detection (data not shown). Final TPH and PAH concentrations were calculated using standard calibration curves for TPH and each PAH.

Monitoring of heterotrophic and hydrocarbon-degrading microbial populations. Total heterotrophic and PAH-degrading microbial population counts were determined on days 0, 21, 45, 90, 155, and 200 using a miniaturized most-probable-number (MPN) technique as described previously (39).

DNA extraction. Soil samples for DNA extraction were collected from each microcosm on days 0, 21, 45, 90, 155, and 200 in sterile Eppendorf tubes and stored at −20°C prior to analysis.

Total community DNA was extracted from all soil microcosms following a bead beating protocol using an Ultraclean DNA soil extraction kit (MoBio Laboratories, Inc., Solano Beach, CA). Suitable yields of high-molecular-weight DNA (5 to 20 μg g of soil−1) were obtained. An additional purification step with a Pure DNA Wizard kit (Promega, Madison, WI) was employed to avoid PCR inhibition.

Preliminary data for independent triplicate microcosms showed that the Pearson correlation moment vector (r) calculated from the relative band intensities of DGGE profiles for treatments 1M, 2M, and 4M (after 0 and 200 days of incubation) was more than 0.85. These data indicated that there was homogeneity in
TABLE 2. Concentrations and removal of TPH and target PAHs in bioremediation treatments

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc (mg kg−1 dry wt of soil)</th>
<th>% Removal from bioremediation treatments at day 200 of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil, day 0</td>
<td>Dry soil, day 200 (1M)</td>
</tr>
<tr>
<td>TPH</td>
<td>8.196 ± 480 A</td>
<td>8.120 ± 285 A</td>
</tr>
<tr>
<td>Aacenaphthene</td>
<td>151 ± 4 C</td>
<td>80 ± 7 A</td>
</tr>
<tr>
<td>Fluorene</td>
<td>182 ± 2 D</td>
<td>137 ± 10 A</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>496 ± 24 A</td>
<td>510 ± 23 A</td>
</tr>
<tr>
<td>Anthracene</td>
<td>114 ± 15 A</td>
<td>85 ± 8 A</td>
</tr>
<tr>
<td>4CIPhe</td>
<td>72 ± 6 A</td>
<td>75 ± 1 A</td>
</tr>
<tr>
<td>3CIPhe</td>
<td>75 ± 4 A</td>
<td>81 ± 2 A</td>
</tr>
<tr>
<td>CI3A</td>
<td>25 ± 5 A</td>
<td>20 ± 1 A</td>
</tr>
<tr>
<td>CYP4/9CIPhe</td>
<td>131 ± 7 A</td>
<td>128 ± 4 A</td>
</tr>
<tr>
<td>1CIPhe</td>
<td>40 ± 1 A</td>
<td>41 ± 2 A</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>693 ± 48 A</td>
<td>728 ± 38 A</td>
</tr>
<tr>
<td>Pyrene</td>
<td>387 ± 30 A</td>
<td>403 ± 9 A</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>108 ± 10 A</td>
<td>115 ± 3 A</td>
</tr>
<tr>
<td>Cryanthracene</td>
<td>144 ± 10 A</td>
<td>153 ± 8 A</td>
</tr>
<tr>
<td>Benzo(b+k)fluoranthene</td>
<td>82 ± 7 A</td>
<td>71 ± 12 A</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>21 ± 2 A</td>
<td>22 ± 1 A</td>
</tr>
<tr>
<td>Total PAHs</td>
<td>2,724 ± 134 C</td>
<td>2,649 ± 130 C</td>
</tr>
</tbody>
</table>

a Different letters indicate significant differences between treatments (P < 0.05).
b Total PAHs determined from the total target PAHs analyzed.

RESULTS

Soil properties. The soil was a loamy clay soil containing 40% clay, 28% silt, and 32% sand. The total organic carbon and total nitrogen contents were 4.2% and 0.15%, respectively; the inorganic nitrogen content was 20 mg kg−1 nitrate and 2.8 mg kg−1 ammonium, and the phosphate content was low (less than 1 mg kg−1). The soil had low conductivity (228 μS cm−1), the pH was 7.5, and the water content was extremely low (1.6%). Conversely, the soil had a high WHC (27.7%), possibly due to the high clay content. The PAH degraders accounted for up to 12% of the total heterotrophic microbial population, with 1.8 × 109 cells g (dry weight) of soil−1.
Total petroleum hydrocarbon and polycyclic aromatic hydrocarbon biodegradation. The contaminated soil had a high initial TPH concentration (around 8,000 mg kg⁻¹ [dry weight] of soil⁻¹) (Table 2). When aromatic compounds were examined, no phenol derivatives, naphthalene, or alkyl derivatives of naphthalene were detected (the detection limit was more than 0.5 μg g⁻¹ [dry weight] of soil⁻¹). Consequently, we presumed that a limited amount of PAHs and TPH was lost by evaporation and/or biodegradation prior to soil sampling. PAHs with three and four rings were the most abundant components, and their concentrations were between 100 and 700 mg kg⁻¹. The polyaromatic fraction accounted for 90% of the TPH fraction (as determined by gravimetric measurement), while the TPH accounted for 85% of the total organic extract.

The TPH concentration did not decrease significantly (*P > 0.05*) in the untreated soil (treatment 1M) over the course of the 200-day experiment, whereas there was a slight decrease in autoclaved soil (treatment 3M). The same behavior was observed for the target PAHs. Hence, untreated dry soil (treatment 1M) was used as a control with which to calculate hydrocarbon biodegradation for the different treatments and to compare changes in microbial diversity.

By the end of the experiment (200 days), significant TPH biodegradation was observed for all treatments (treatments 2M and 4M to 7M) (Table 2). Biostimulation without nutrient addition (treatment 2M) resulted in slightly greater TPH depletion (*P < 0.05*) than the treatments in which nutrients were added (treatments 4M to 7M).

To study the rates of biodegradation of TPH and target PAHs, biphasic TPH degradation kinetics were assessed because a single first-order decay curve fitted to the entire data set did not adequately explain (r² < 0.8) the changes in TPH concentration observed during the course of the experiment. Thus, two degradation rates (k₁ and k₂) were defined according to the breakpoints that best fit the data for all treatments; k₁ was used for the early-stage group (from 0 to 45 days of incubation), and k₂ was used for the late-stage group (from 90 to 200 days of incubation) (Table 3). It is important to note that k₂ was 1 order of magnitude lower than k₁ for all treatments, with values near zero for treatments with nutrients. During the first 45 days, the first-order TPH degradation rate constant (k₁) was slightly lower for the treatment without nutrients (treatment 2M) than for the other treatments. In contrast, the rate constant (k₂) for the late stage (90 to 200 days) was higher (*P < 0.05*). These rate constants are similar to those reported in other studies of bioremediation in hydrocarbon-contaminated soils (18, 35).

Two- and three-ring PAHs were highly degraded (80 to 100%) during the first 45 days, and the biodegradation rate (k) was the same for all treatments (*P > 0.05*) (Tables 2 and 3). An exception was 1-methyl-anthracene, for which the biodegradation rate was higher (*P < 0.05*) in treatments without nutrient amendment. Interestingly, phenanthrene and anthracene were not completely degraded (Table 2). One possible explanation for this could be the low bioavailability of the residual quantities of these compounds present after day 45 (31). The biodegradation kinetics of fluoranthene were not affected by nutrient addition (Table 3), whereas the early-stage biodegradation kinetics (k₁) of pyrene were slower in the absence of nutrient addition (*P < 0.05*).
The degree of benzo(a)anthracene and chrysene degradation was significantly different when biostimulation without addition of nutrients (treatment 2M) was compared with treatments in which nutrients were added (treatments 4M to 7M). Both compounds were degraded more \((P < 0.05)\) under biostimulation conditions without nutrient addition than in treatments with nutrient addition (Table 2). Furthermore, the late-stage biodegradation rates were significantly higher \((P < 0.05)\) without nutrient addition [10-fold higher for benzo(a)anthracene biodegradation and threefold higher for chrysene biodegradation] (Table 3). Benzo(a)pyrene and benzo(b+k)fluoranthene were not significantly degraded \((P > 0.05)\). The percentages of PAH biodegradation and the kinetics are similar to those reported in other studies (7, 12).

**Monitoring of the heterotrophic and polycyclic aromatic hydrocarbon-degrading microbial populations.** As shown in Fig. 1, the sizes of both the heterotrophic and PAH-degrading microbial populations increased 2 to 3 orders of magnitude between day 0 and day 21 for treatments in which there was nutrient addition (treatments 4M to 7M), whereas the population subjected to the biostimulation treatment without nutrient addition (treatment 2M) increased more slowly (1 to 2 orders of magnitude at 21 to 45 days). After 90 days of incubation, the sizes of both populations returned to the initial values for treatments in which there was nutrient addition (treatments 4M to 7M), whereas for treatment 2M the PAH-degrading population remained 1 order of magnitude larger than the population at the initial stage. In untreated dry soil (treatment 1M), the size of the heterotrophic population remained constant until day 135 and the size of the PAH-degrading population remained constant until day 90. Between days 135 and 200 the sizes of the two populations decreased by approximately 1 order of magnitude.

Aeration and the optimal humidity (treatment 2M) resulted in a remarkable shift in the proportion of the PAH-degrading population (Fig. 1).

No differences in microbial population counts were observed when the results for addition of biosurfactant (treatment 5M), addition of ferric octoate (treatment 7M), or bioaugmentation (treatment 6M) were compared with the results for treatment 4M.

**Analysis of treatments by denaturing gradient gel electrophoresis.** The biodegradation results and microbial counts revealed three different treatment groups: (i) untreated soil, (ii) treated soil without nutrient addition, and (iii) treated soil with nutrient addition. Thus, the effects of different biostimulation conditions on the structure and dynamics of the bacterial community were analyzed by DGGE by using samples from treatments 1M (untreated soil), 2M (aerated soil at 40% water-holding capacity without nutrient addition), and 4M (like treatment 2M but with nutrient addition) taken during the 200-day experiment. In addition, the effect of bioaugmentation (treatment 6M) was monitored by DGGE, and this treatment was compared with treatments 4M and 1M.

The bacterial community in untreated soil (treatment 1M) was complex (27 or 28 DGGE bands) and did not change over the course of the 200-day experiment (Fig. 2). However, biostimulation treatments with (treatment 4M) and without (treatment 2M) nutrient addition resulted in shifts in the composition of the microbial community. In particular, the initial profiles (21 and 45 days) were clearly different; the 2M and 4M treatments produced 22 to 24 and 19 to 22 DGGE bands, respectively (Fig. 2).

The microbial diversity decreased during the maximum biodegradation period for all treatments, from 1.28 on day 0 to 1.18 after 90 days, and remained low until the end of the incubation. Other authors have described both increases and reductions in diversity during biodegradation. Our results are similar to those reported by Andreoni et al. (6) obtained with a PAH-contaminated soil, whereas Kaplan and Kitts (18) and Zucchi et al. (41) reported increases in diversity during bioremediation of crude oil-contaminated soil.

**Principal-component analysis of the bacterial community.** Temporal changes in the bacterial community were monitored using principal-component analysis (Fig. 3). Based on visual inspection of the raw data, there was a clear difference in the DGGE profiles between microcosms that depended on the time and type of biostimulation treatment. PCA revealed three different groups; the first group consisted of untreated soil (at 200 days), and the second and third groups consisted of biostimulated soil with and without nutrient addition, respectively. Both biostimulation treatments (treatments 2M and 4M) exhibited a noticeable and regular separation from untreated soil (treatment 1M) in the first principal component (PC1), which explained 35.9% of the variation in the data.
during the 200 days of incubation (Fig. 3). Furthermore, for each treatment the PC1 value was increasingly far from the initial point as incubation time increased. The highest second principal component (PC2) for the 2M treatment corresponded to positive values, while for the 4M treatment the values were negative (Table 4). This could explain the distance in PC2 observed between the biostimulation treatments. Analysis of variance of the PC2 scores, which represented 24.0% of the variation in the data, showed that there was a significant difference between biostimulation groups ($P < 0.05$) over the course of the experiment. Coincidentally, the greatest change in PC2 occurred during the first 45 days of incubation, during which the TPH biodegradation rates were more significant (Table 2).

It is interesting that only two bands were shared by different treatments. Bands B2-11 and B2-20 were identical to bands B4-12 and B4-24, respectively. Furthermore, these DGGE bands displayed similar dynamics for the two treatments and exhibited the most negative PC1 loading values (0.909 and 0.961, respectively). Their dynamics could explain the migration along PC1 observed for both treatments. The treatment 2M bands with the highest PC2 loading values were B2-3 and B2-16, while for the 4M treatment B4-17 had the most negative
No nutrients (2M)\(^a\)
- B2-1 (135–200 days) \(\rightarrow\) AY758563 0.490 0.248 98.4 Sphingomonas yanoikuyae Q1 (OS37525) Sphingomonadaceae (α)
- B2-3 (90–200 days) \(\rightarrow\) AY758564 0.390 0.826 97.7 Environmental CEB clone (AY038871) Sphingobacteriaceae (CFB group)
- B2-5 \(\rightarrow\) AY758565 0.012 0.596 95.4 Cytophaga sp. strain MDA2507 (AY238333) Flavobacteriaceae (CFB group)
- B2-7 \(\rightarrow\) AY758566 0.565 0.245 96.7 Uncultured bacterium (AF465688) Unclassified γ-Proteobacteria
- B2-8 (21–45 days) \(\rightarrow\) AY758567 0.399 0.297 98.5 Acidovorax sp. strain UFZ-B517 Comamonadaceae (β)

B2-9\(^a\) (21–90 days) \(\rightarrow\) AY758568 0.467 0.299 97.1 Xanthomonas axonopodis (AF447242) Xanthomonadaceae (γ)
- B2-11* (21–90 days) \(\rightarrow\) AY758569 0.909 0.377 96.7 Spiroplana sp. strain C06 (SC520146) Sphingomonadaceae (α)
- B2-12* (21–90 days) \(\rightarrow\) AY758570 0.477 0.643 98.4 Sphingomonas sp. strain C06 (SC520146) Sphingomonadaceae (α)
- B2-13 \(\rightarrow\) AY758571 0.189 0.531 96.4 Sphingomonas sp. strain JSI (SPP427917) Sphingomonadaceae (α)
- B2-14* \(\rightarrow\) AY758572 0.189 0.531 98.6 Environmental clone AP-16 (AY145553) Rhizobiales (α)
- B2-15* (45–200 days) \(\rightarrow\) AY758573 0.882 0.186 94.5 Environmental clone (AB074615) Xanthomonadaceae (γ)
- B2-16* (45–200 days) \(\rightarrow\) AY758574 0.646 0.644 99.6 Environmental clone (AB074615) Xanthomonadaceae (γ)
- B2-17* \(\rightarrow\) AY758575 0.813 0.086 99.8 Environmental clone (AY130099) Rhodospirillaceae (α)
- B2-18* \(\rightarrow\) AY758576 0.085 0.657 97.3 Sphingomonas sp. (X72723) Sphingomonadaceae (α)
- B2-19 (90–200 days) \(\rightarrow\) AY758577 0.630 0.199 97.2 Environmental clone C5-K12 (UBA421218) Unclassified γ-Proteobacteria
- B2-20* (21–45 days) \(\rightarrow\) AY758578 0.961 0.100 99.8 Azospirillum sp. strain C06 (AY129799) Rhodospirillaceae (α)
- B2-22 \(\rightarrow\) AY758579 0.166 0.547 97.3 Roseomonas genomspecies (AY150049) Roseomonadaceae

Nutrient addition (4M)
- B4-3 (45 days) \(\rightarrow\) AY758580 0.097 0.278 100 Sphingomonas sp. strain S37 (AF367204) Sphingomonadaceae (α)
- B4-4 \(\rightarrow\) AY758581 0.643 0.538 94.7 Brevundimonas alba H12CS (AF926688) Unclassified α-Proteobacteria
- B4-5 \(\rightarrow\) AY758582 0.675 0.337 99.4 Tropjanella thalesunica (AF680496) Rhodospirillaceae (α)
- B4-6 (21–45 days) \(\rightarrow\) AY758583 0.565 0.458 98.5 Agrobacterium tumefaciens (ATU295683) Rhizobiales (α)
- B4-7 (21 days, 90–200 days) \(\rightarrow\) AY758584 0.399 0.297 97.5 Alcaligenes sp. (AS168591) Alcaligenaceae (β)
- B4-8* (21–45 days) \(\rightarrow\) AY758585 0.407 0.299 98.9 Xanthomonas sp. strain V4.BO.41 (AY244722) Xanthomonadaceae (γ)
- B4-10 (65–135 days) \(\rightarrow\) AY758586 0.342 0.667 93.9 Environmental clone (AJ357272) Unclassified γ-Proteobacteria
- B4-11* (21–45 days) \(\rightarrow\) AY758587 0.597 0.559 99.8 Sphingomonas herbicidivorans (AB022428) Sphingomonadaceae (α)

- B4-12* (21–45 days) \(\rightarrow\) AY758588 0.909 0.337 95.3 Sphingomonas alchaeus (SA165R3D) Sphingomonadaceae (α)
- B4-13 (45–90 days) \(\rightarrow\) AY758589 0.435 0.003 92.7 Erythrobacter sp. (AB011075) Sphingomonadaceae (α)
- B4-14* (135–200 days) \(\rightarrow\) AY758590 0.263 0.669 95.1 Xanthomonas sp. strain V4.BO.41 (AY244722) Xanthomonadaceae (γ)
- B4-16 (90 days) \(\rightarrow\) AY758591 0.189 0.531 99.8 Achromobacter xylosidans (AE225979) Alcaligenaceae (B)
- B4-17 (90–200 days) \(\rightarrow\) AY758592 0.519 0.787 95.0 Xanthomonas sp. strain V4.BO.41 (AY244722) Xanthomonadaceae (γ)
- B4-20 (21–200 days) \(\rightarrow\) AY758593 0.882 0.186 98.8 Achromobacter sp. strain LM05911 Alcaligenaceae (B)
- B4-21 (90–135 days) \(\rightarrow\) AY758594 0.646 0.644 96.3 Xanthomonas sp. strain V4.BO.41 (AY244722) Xanthomonadaceae (γ)
- B4-23 (135–200 days) \(\rightarrow\) AY758595 0.630 0.199 97.8 Sinorhizobium fredii USDA257 (AY260100) Rhizobiales (α)
- B4-24* (21–45 days) \(\rightarrow\) AY758596 0.961 0.100 99.8 Azospirillum sp. strain C06 (AY129799) Rhodospirillaceae (α)
- B4-25 \(\rightarrow\) AY758597 0.514 0.679 90.1 Erythrobacter sp. strain AS-45 (ESP91206) Roseomonas genomspecies strain ATCC 49961 (AY150050) Roseomonadaceae
- B4-27 \(\rightarrow\) AY758598 0.586 0.666 93.2 Rhodococcus sp. strain RH45 (RS16316) Nocardiaceae (high-G+C-content gram-positive bacteria)

PC2 loading value. Band B4-3, which was the most abundant band in the early biodegradation stage at 0 to 45 days for the 4M treatment, disappeared after 90 days and did not have a high PCA loading value (Table 4). Nevertheless, this band could be important in the biodegradation of three-ring PAHs because at this time these compounds exhibited maximum depletion. **Phylogenetic and dynamic analysis of excised bands.** Taxonomic assignments of the prominent DGGE bands were determined for biostimulation treatments 2M and 4M. Bands from untreated soil (treatment 1M) were not excised because they were weak, but they were assigned to comigrating bands of treatments 2M and 4M which exhibited higher intensity (Fig. 2 and Table 4). Table 4 shows the closest relatives of DGGE bands excised for biostimulation treatments 2M and 4M. Most of the sequences derived from DGGE bands exhibited levels of similarity greater than 95%. Taking into account the assignment to comigrating bands, the original soil contained five α-Proteobacteria (Sphingomonas, Rhizobium-Agroberctium, and Azospirillum groups) and eight γ-Proteobacteria (Xanthomonas group), according to RDP classification (24). Biostimulated soil from treatment 2M...
contained 53% α-Proteobacteria (9/17 DGGE bands), 6% β-Proteobacteria (1/17 DGGE bands), and 23% γ-Proteobacteria (4/17 DGGE bands) (Table 4). No rhodococci were found, but the closest relatives to the Cytophaga-Flavobacterium-Bacteroides (CFB) group, which accounted for 12% of the sample (2/17 DGGE bands), were found after 45 days. Soil subjected to the 4M treatment contained 55% α-Proteobacteria (11/20 DGGE bands), and 5% high-G+C-content gram-positive bacteria were found at early stages of biodegradation.

At early stages (0 to 90 days), for treatment 2M soil five dominant bands (relative intensity greater than 5%) were observed (B2-11, B2-12, B2-20, B2-8, and B2-9) (Table 4). Three of these bands (B2-11, B2-12, and B2-20) belonged to α-Proteobacteria (Sphingomonadaceae and Rhodospirillaceae), whereas B2-8 belonged to β-Proteobacteria (Comamonadaceae) and B2-9 belonged to γ-Proteobacteria (Xanthomonadaceae). Neither CFB bacteria nor high-G+C-content gram-positive bacteria were found at early stages of biodegradation.

At late stages (90 to 200 days), for treatment 4M soil nine dominant bands (relative intensity greater than 5%) were observed (B4-7, B4-10, B4-14, B4-20, B4-21, B4-23, and B4-28). Four of these bands (B4-10, B4-14, B4-17, and B4-21) belonged to γ-Proteobacteria (Xanthomonadaceae and unclassified group), two bands (B4-20 and B4-7) belonged to β-Proteobacteria (Alcaligenaceae), and one band (B4-23) belonged to α-Proteobacteria (Rhizobiaceae), whereas band B4-28 belonged to the high-G+C-content gram-positive bacteria (Nocardiaceae). No CFB bacteria were found.

Phylogenetic groups during bioremediation. At the beginning of the experiment, α-Proteobacteria (78%), dominated by Sphingomonadaceae, were predominant in contaminated soil, followed by γ-Proteobacteria (16%), dominated by Xanthomonadaceae, whereas the other groups were scarce. During biodegradation, the microbial population shifted dramatically, and after 200 days of incubation of the 2M treatment, γ-Proteobacteria were dominant (50%), followed by α-Proteobacteria (25%) and the CFB group (22%), while for the 4M treatment, α-Proteobacteria and γ-Proteobacteria were the dominant groups (36% each), followed by β-Proteobacteria and high-G+C-content gram-positive bacteria (19% and 8%, respectively) (Fig. 4).

A correlation analysis of the phylogenetic groups and evolution of the TPH concentration showed that the α- and γ-Proteobacteria exhibited the highest positive and inverse correlations with the TPH concentration, respectively ($r^2 = 0.92$ to 0.93) for the 2M treatment; for the 4M treatment the α-Proteobacteria exhibited the highest positive correlation ($r^2 = 0.78$), whereas the β-Proteobacteria exhibited the highest inverse correlation ($r^2 = 0.85$), followed by the γ-Proteobacteria ($r^2 = 0.78$). For target PAH concentrations in the 2M treatment, the CFB group exhibited the highest inverse correlation ($r^2 = 0.81$ to 0.85) with the pyrene, benzo(a)anthracene, and chrysene concentrations, whereas α-Proteobacteria and γ-Proteobacteria exhibited the highest positive and inverse correlations with fluoranthene ($r^2 = 0.92$ to 0.94), phenanthrene ($r^2 = 0.76$), and anthracene ($r^2 = 0.84$). β-Proteobacteria did not exhibit good correlations with target PAHs ($r^2 = 0$ to 0.4). In contrast, for nutrient treatment 4M, α-Proteobacteria and β-Proteobacteria exhibited the highest positive and inverse cor-
relations, respectively, with fluoranthene and pyrene ($r^2 = 0.83$ to 0.89). Also, $\beta$-Proteobacteria exhibited a good inverse correlation with phenanthrene, anthracene, benzo(a)anthracene, and chrysene ($r^2 = 0.84$ to 0.92). $\gamma$-Proteobacteria exhibited lower correlations with PAH concentrations ($r^2 = 0.5$ to 0.7) for the 4M treatment.

**DISCUSSION**

A creosote-contaminated soil from a wood treatment facility that had been operating for at least 30 years was the subject of the present study. A high proportion of PAH-degrading bacteria at the beginning of the experiment (Fig. 1) and the absence of a lag phase at the beginning of TPH degradation (data not shown) revealed the presence of a preadapted PAH-degrading microbial population in the original soil.

**Effects of different treatments on the degradation of polycyclic aromatic hydrocarbons.** The kinetics of TPH degradation displayed a biphasic pattern for all bioremediation treatments. This behavior may have been due to a number of factors, such as a decrease in PAH bioavailability (3), accumulation of toxic PAH metabolites (11), or enrichment of more recalcitrant compounds (30). In a parallel study, a decrease in bioavailability was demonstrated (31). The accumulation of metabolites resulting from oxidation of PAHs can reduce the viability of several PAH degraders in addition to inhibiting the degradation of PAH (11, 20, 21). Nevertheless, this probably was not the case in this study, because polar metabolites determined by gas chromatography-mass spectrometry of the derivatized polar fraction did not accumulate in the soil (31). In addition, leachates from the soil after the different treatments showed that there was a reduction in soil toxicity, as determined by Microtox (31).

Taking into account the finding that in the original dry soil (water content, 1.6%) the TPH concentration was not reduced during the experiment, water content and aeration became the key factors for soil bioremediation. The quantities of nutrients added in our treatments, taking into account only the inorganic nitrogen forms, were established in order to obtain a molar C/N/P ratio of 300:10:1. The required quantities were added at three times during the experiment in order to avoid inhibition caused by a drastic osmotic change, as has been described elsewhere (38). Recently, Atagana (7) described the ratio used in this study as optimal for a creosote-contaminated soil.

As shown in Table 3, our results suggest that nutrient addition had a negative effect on late-stage biodegradation of four-ring PAHs and TPHs. The presence of an excess of nutrients...
could have inhibited the biodegradation of high-molecular-weight PAHs. In fact, Rhykerd et al. (27) described a reduction in bioremediation of a motor oil due to an increase in the salinity produced by NaCl, and Braddock et al. (10) attributed a reduction in the microbial activity of an Arctic hydrocarbon-contaminated soil to the salinity produced by an excess of nutrients.

In a recent study, Roling-Wilfred et al. (29) suggested that the addition of nutrients implies that there are differences in hydrocarbon biodegradation rates and variations in the microbial population. Therefore, for the present study, the differential microbial population shifts due to different inorganic nutrient contents may also explain the different biodegradation capabilities observed for biostimulation treatments with and without nutrient addition.

Response of the microbial population to bioremediation of polycyclic aromatic hydrocarbons. At early stages (0 to 45 days) of the bioremediation process, the addition of nutrients caused significant increases in both the heterotrophic and PAH-degrading microbial populations, while soil stimulated only by aeration and optimum humidity exhibited smaller increases in the sizes of both populations and a marked delay in the increase in microbial PAH degraders (Fig. 1). This difference in behavior is consistent with the kinetics of TPH depletion observed during the early stage (Table 2). As shown in Table 2, a higher TPH degradation rate was observed for treatments to which nutrients were added. In contrast, the proportion of the PAH-degrading microbial population compared to the heterotrophic population for the treatment without nutrients increased noticeably at 21 and 45 days and reached 100% at 135 days, while lower proportions were observed for treatments with nutrients (Fig. 1). In addition, at the end of the experiment a larger PAH-degrading microbial population was observed for the treatment without nutrients. Thus, the majority presence of microbial PAH degraders during the late stage could be linked to the higher TPH degradation rates observed, especially with more recalcitrant PAHs. Both changes in PAH bioavailability and enrichment of more recalcitrant PAHs could change the microbial population and the multisubstrate interactions (8, 9, 14, 22).

We found 13 different genera that were related to DGGE excised bands during biostimulation of a heavily PAH-contaminated soil: Sphingomonas, Azospirillum, Roseomonas, Brevedimonas, Trojanella, Agrobacterium, Alcaligenes, Xanthomonas, Achromobacter, Sinorhizobium, Erytrobacter, Cytophaga, and Rhodococcus.

During the first stage of biodegradation in the 2M treatment and the 4M treatment, the Sphingomonas and Azospirillum phylotypes (α-Proteobacteria) were the dominant taxa (Table 4). Furthermore, the only two coincident DGGE bands for the two treatments belong to these phylotypes (B2-11 = B4-12 and B2-20 = B4-24). Therefore, taking into account that with both treatments high levels of TPH and PAH biodegradation were observed during the first 90 days, these phylotypes may be linked to the fast biodegradation observed for acenaphthene, fluorene, phenanthrene, anthracene, alkyl-phenanthrenes, fluoranthene, and pyrene. These results are consistent with those of Roling-Wilfred et al. (29) and Kasai et al. (19), who observed strong dominance of α-Proteobacteria in bacterial communities found in recently spilled oil in Nak-hodka and in oil-spiked intertidal sediments after 6 days, respectively.

During data analysis, a high abundance of gram-negative bacteria was observed that could have been due to the extraction bias. However, the extraction method used in this study was able to extract DNA from gram-positive bacteria, such as Rhodococcus (this study), Mycobacterium, and Bacillus laboratories, suggesting that gram-negative bacteria dominated the creosote-contaminated soil during the bioremediation process.

Bioaugmentation assay. For the bioaugmentation treatment (treatment 6M), no differences in the rates of biodegradation of TPH and PAHs or in community structure determined by PCR-DGGE and MPN analysis were detected. In a previous study (36) we observed that the PAH-degrading capability of consortium AM (used as the inoculum in the present study) was not affected after growth in rich media. Also, this consortium contained 19 microbial components identified as Sphingomonas, Pseudomonas, Stenotrophomonas, Ocrobactrum, Alcaligenes, Pandorea, Labrys, and Fusarium (37). As shown in Fig. 5, none of the visible bands from consortium AM comigrated with bands from inoculated soil (treatment 6M), indicating that the inoculated microorganisms did not compete favorably with the indigenous bacterial community, even though they were originally from a PAH-contaminated soil (36) and the inoculum size was at least equal to (10^6 MPN g of soil^-1), if not larger than, the indigenous population and organisms were periodically inoculated throughout the experiment (Table 1). Moreover, the microbial population structure of inoculated soil was very similar to that of the soil biostimulated with nutrients during the first 45 days, in which the maximum biodegradation rates were observed. Therefore, the indigenous population changed independent of the inoculum, indicating that the biodegradation process was probably the strongest selection factor for the shifts in the microbial population.

Conclusions. This study showed that specific phylotypes of bacteria are related both to inorganic nutrient addition to the soil and to different phases of PAH degradation in a nonspiked PAH-contaminated soil, and it highlighted the importance of understanding how interspecies interactions, nutrient effects, and changes in PAH bioavailability and recalcitrance influence the degradation capability and structure of a microbial community.

ACKNOWLEDGMENTS

This research was funded by grants from the Spanish Government’s National Plan for Research (REN2001-3425/TECNO; PPO-2000-0105-P4 and 2001SGR 00143) and CIRIT 2001SGR 00143. M.V. received a postgraduate fellowship from the Comisionat de Recerca i Innovacio Tecnologica (CIRIT) of the Generalitat de Catalunya. We declare that the experiments discussed in this paper complied with current Spanish law.

REFERENCES
