CORE
Metadata, citation and similar papers at core.ac.uk
Provided by HAL-uB

Betaproteobacteria dominance and diversity shifts in the bacterial community of a PAH-contaminated soil exposed to phenanthrene.

Florence Martin, Stéphane Torelli, Denis Le Paslier, Agnès Barbance, Fabrice Martin-Laurent, David Bru, Roberto Geremia, Gérard Blake, Yves Jouanneau

To cite this version:

Florence Martin, Stéphane Torelli, Denis Le Paslier, Agnès Barbance, Fabrice Martin-Laurent, et al.. Betaproteobacteria dominance and diversity shifts in the bacterial community of a PAH-contaminated soil exposed to phenanthrene.. Environmental Pollution, Elsevier, 2012, 162, pp.345-53. <10.1016/j.envpol.2011.11.032>. <hal-01063697>

HAL Id: hal-01063697

https://hal.archives-ouvertes.fr/hal-01063697

Submitted on 15 Sep 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

*Manuscript
Click here to download Manuscript: Martin et alEP1.doc
Click here to view linked References

Betaproteobacteria dominance and diversity shifts in the bacterial community of a PAH-contaminated soil exposed to phenanthrene

Florence Martin[a,b], Stéphane Torelli[a,b], Denis Le Paslier[c,d,e], Agnès Barbance[c,d,e], Fabrice Martin-Laurent[f], David Bru[f], Roberto Geremia[g], Gérard Blake[h] and Yves Jouanneau[a,b*]

[a]CEA, DSV, iRTSV, Laboratoire de Chimie et Biologie des Métaux, F-38054 Grenoble.
[b]UJF-Grenoble 1/CNRS, UMR 5249, F-38041, Grenoble, France

[c] CEA, DSV, Institut de Génomique, Genoscope, 2 rue Gaston Crémieux, F-91057 Evry.
[d] CNRS-UMR 8030 F-91057 Evry.
[e] UEVE, Université d'Evry, F-91057 Evry, France

[f]INRA, Université de Bourgogne, UMR 1229 Microbiologie du Sol et de l'Environnement, BP 86510, 21065 Dijon Cedex, France

[g]UJF-Grenoble 1/CNRS, UMR 5553 Laboratoire d'Ecologie Alpine, F-38041 Grenoble, France

[h]LOCIE, Université de Savoie, F-73376 Le Bourget-du-Lac, France

[*]Corresponding author: Yves Jouanneau, LCBM/iRTSV, CEA-Grenoble, F-38054 Grenoble Cedex 9, France. Tel. : 33 4 38 78 43 10; Fax : 33 4 38 78 54 87
yves.jouanneau@cea.fr

Abstract

In this study, the PAH-degrading bacteria of a constructed wetland collecting road runoff has been studied through DNA stable isotope probing. Microcosms were spiked with ^{13}C-phenanthrene at 34 or 337 ppm, and bacterial diversity was monitored over a 14-day period. At 337 ppm, PAH degraders became dominated after 5 days by Betaproteobacteria, including novel *Acidovorax*, *Rhodoferax* and *Hydrogenophaga* members, and unknown bacteria related to Rhodocyclaceae. The prevalence of Betaproteobacteria was further demonstrated by phylum-specific quantitative PCR, and was correlated with a burst of phenanthrene mineralization. Striking shifts in the population of degraders were observed after most of the phenanthrene had been removed. Soil exposed to 34 ppm phenanthrene showed a similar population of degraders, albeit only after 14 days. Our results indicate that specific Betaproteobacteria are involved in the main response to soil PAH contamination, and illustrate the potential of SIP approaches to identify soil PAH degraders.

Key words : 16S rRNA sequences; stable isotope probing; PAH degradation; phenanthrene; Betaproteobacteria

Capsule : On a site collecting road runoff, implementation of stable isotope probing to identify soil bacteria responsible for phenanthrene degradation, led to the discovery of new Betaproteobacteria distantly related to known PAH degraders.

Introduction

Among the environmental pollutants that perturb ecosystems and threaten human beings, polycyclic aromatic hydrocarbons (PAHs) are of special concern because they are persistent and accumulate along the trophic chain. To clean up contaminated sites, bioremediation strategies have been proposed based on the ability of particular microorganisms to degrade PAHs (Doyleet al., 2008). Numerous bacterial isolates able to utilize PAHs as carbon sources have been described, and the biochemical pathways responsible for their oxidative degradation have been investigated (Penget al., 2008). Based on their frequent occurrence on polluted sites and their metabolic potential, members of the Sphingomonadaceae and Actinobacteria are considered potent PAH degraders in soil or sediments (Leyset al., 2004; Leyset al., 2005; Alonso-Gutierrezet al., 2009). However, in the last decade, culture-independent studies highlighted the great bacterial diversity in environmental ecosystems such as soils, and showed that culturable bacteria represented less than 5 % of the existing species (Coleet al., 2010). Hence, PAH-degrading strains described so far might not be representative of soil bacteria that actually remove pollutants in situ. Consistent with this idea, investigations involving a selective labeling of the bacteria of interest through stable-isotope probing (SIP (Radajewskiet al., 2000)) led to the discovery of new bacteria involved in pollutant degradation (Wackett, 2004). SIP-based methods permit the exploration of uncultured bacteria present in natural or contaminated environments and reduce biases associated with selection in artificial media (Dumont and Murrell, 2005). Using a field-based SIP strategy to track naphthalene degraders on a coal tar contaminated site, Jeon *et al*. identified a novel *Polaromonas* strain, as the major players (Jeonet al., 2003). In studies targeting naphthalene-, phenanthrene- or pyrene-degrading bacteria in a bioreactor treating PAH-contaminated soil, different bacterial taxa were detected depending on the PAH-substrate used as probe (Singletonet al., 2005; Singletonet al., 2006). An *Acidovorax* strain,

71 which was found to be dominant among phenanthrene-degrading bacteria, was later isolated
72 and characterized as a novel PAH degrader (Singletonet al., 2009). Recently, bacteria related
73 to the *Pseudoxanthomonas* and *Microbacterium* genera were identified as the main
74 phenanthrene degraders in soil but diversity changes were observed in soil also treated with
75 root exudates (Cebronet al., 2011). In another SIP-based study targeting anthracene-degrading
76 bacteria, dominant soil degraders were found to be affiliated to the *Sphingomonadales* and
77 *Variovorax* taxa (Joneset al., 2011).
78 In the present study, we have implemented a SIP strategy to explore the diversity of a PAH-
79 degrading community in a constructed wetland collecting the road runoff from a highway.
80 Using ^{13}C-phenanthrene as tracer, we examined changes in the soil PAH-degrader population
81 as a function of the dose and time of exposure to the tracer. For this purpose, soil DNA was
82 extracted after various SIP treatments, separated by isopycnic centrifugation, and labeled
83 DNA were used to identify phenanthrene degraders based on 16S rRNA gene sequences. .
84 Moreover, dose- and time-dependent changes in the composition of the phenanthrene-exposed
85 bacterial community were monitored by a combination of molecular methods, including
86 single-strand conformation polymorphism and quantitative PCR. Results revealed that, in
87 addition to *Burkholderiales*, bacteria related to the Rhodocyclaceae and *Thiobacillus* taxa
88 appeared as new potential phenanthrene degraders. Our data also provide new insights into
89 the response of a large panel of specific degraders, mostly undescribed, to soil PAH
90 contamination.
91

MATERIALS AND METHODS

Sampling site and determination of soil PAH concentration

Soil samples were obtained from a constructed wetland collecting road runoffs from a highway near Chambéry (France) in October 2008. The facility, administered by the AREA company, was colonized by *Typha latifolia* and *Phragmites australis* all over the structure. Soil was sampled from the 10-cm upper layer, sieved to about 3 mm and stored in a closed plastic box at 4°C until use. The physico-chemical composition of the soil used will be given elsewhere.

Synthesis of [U-^{13}C]-phenanthrene

[U-^{13}C]-phenanthrene was prepared from [U-^{13}C] succinic anhydride and [U-^{13}C] naphthalene (both from Sigma-Aldrich), according to a previously described method (Singleton, et al., 2005) and references cited therein) with minor modifications. The product was obtained in five steps with a yield of approximately 44%. The purity and homogeneity of the final preparation were checked by ^{1}H NMR in $CDCl_3$ (Fig. S1) and GC-MS (m/z = 192 (M^+)). A detailed description of the preparation procedure is available upon request.

Microcosm setup and incubation conditions

Incubations were performed in 250 ml sterilized glass Erlenmeyer flasks, closed with rubber stoppers. Microcosms consisted of 20 g wet soil (water content, 59 %, vol/wt) and 5 ml of a salt solution (7.5 mM $(NH_4)_2SO_4$ – 20 mM KH_2PO_4 – 30.6 mM Na_2HPO_4 – 0.18 mM $CaCl_2$ – 3.6 10^{-2} mM $FeSO_4$ – 0.81 mM $MgSO_4$), added to favor bacterial metabolism. [U-^{13}C]-phenanthrene was supplied as a 54.9 mM stock solution in dimethylsulfoxide (DMSO) to give final concentrations of either 33.7 or 337 ppm (on a dry wt basis), equivalent to levels 200- or

2000-fold as high as that of phenanthrene initially found in soil. Relevant microcosm experiments were referred to as SIP200 and SIP2000, respectively. Two microcosms supplied with 337 ppm labeled phenanthrene were inoculated with approx. 2 10^8 cells (0.2 ml) of a washed culture of *Sphingomonas* CHY-1 (Willison, 2004). Three types of control flasks were also prepared, which contained either unlabeled phenanthrene (added at 34 ppm as for SIP200), no additional carbon source, or sodium azide (7.7 10^{-5} mol/g dry soil; abiotic control). Microcosms containing [U-^{13}C]-phenanthrene (SIP200 and SIP2000) were prepared in 9 replicates (triplicates at 3 time points), whereas controls with unlabelled phenanthrene or microcosms inoculated with strain CHY-1 were performed in 6 replicates (duplicates at 3 time points). Other control flasks were incubated in duplicates. Flasks were incubated at 25°C in static mode in a dark room.

PAH extraction from soil and quantification

Soil samples (1g) were mixed with 1g anhydrous sodium sulfate and 10 mL hexane in 30-mL glass tubes closed with Teflon-sealed screw-caps. Suspensions were homogenized by Vortex mixing and treatment in an ultrasonic bath for a total time of 1 h. Soil samples were extracted once more with hexane and organic phases were combined, centrifuged for 12 min at 7400 *g* before evaporation to dryness under argon. Dry extracts were dissolved in 0.5 ml acetonitrile. Residual ^{13}C-phenanthrene was extracted in the same way.

Quantification of the 16 priority PAHs was performed with a HP6890 gas chromatograph coupled to a HP5973 mass spectrometer (Agilent Technologies). PAHs were separated on a MDN12 column (30 m by 0.25 mm, 25µm film thickness; Supelco) as previously described (Krivoboket al., 2003). The mass detector was operated in the single-ion monitoring mode. PAHs were eluted between 8.9 and 32.7 min. Concentrations were determined using calibration curves obtained from dilutions of a standard mixture of the 16 PAHs (0.1 mg/ml,

142 Agilent Technologies). ^{13}C-phenanthrene was quantified from the area of the peak detected at
143 m/z : 192 using unlabelled phenanthrene as a standard.
144
145 **Monitoring of mineralization by GC/MS analysis**
146 Quantification of CO_2 evolved in the headspace of microcosms was performed by GC/MS
147 analysis on 25 µl samples of the gas phase withdrawn with a gastight syringe. Analysis was
148 carried out on the same apparatus as above equipped with an HP-PLOT/Q column (15 m by
149 0.32 mm, 20 µm film thickness; Agilent Technologies) with helium as carrier gas maintained
150 at a flow rate of 36 ml/min. The GC was run in the isothermal mode at 40°C with a split ratio
151 of 12:1. $^{13}CO_2$ (m/z = 45) or $^{12}CO_2$ (m/z = 44) were measured with the MS operated in the
152 single-ion monitoring mode. Concentrations were determined from peak area using a
153 calibration curve in the 0 to 200 µM range made with known mixtures of CO_2 in argon. Net
154 $^{13}CO_2$ produced in microcosm headspaces was calculated by subtracting background $^{13}CO_2$
155 present in the air.
156
157 **SSCP fingerprint analyses of 16S rRNA genes**
158 For each tested experimental condition, 1g of soil (wet weight) was sampled and divided into
159 three 250-mg soil aliquots, thus making 3 replicates per flask. DNA was extracted using the
160 Power Soil extraction kit (MO BIO Laboratories). DNA samples were adjusted to 10 ng/µl
161 and used as templates to amplify the V3 region of the 16S rRNA genes. PCR conditions and
162 subsequent capillary electrophoresis were performed as previously described (Zingeret al.,
163 2007). SSCP profiles were computed and normalized. Nonmetric multidimensional scaling
164 (NMDS) ordination was performed with the R software, using the vegan package (R
165 Development Core Team, 2007). Environmental fitting was performed to evaluate the effects
166 of time as a variable, and phenanthrene as a factor, on the bacterial community.

DNA extraction and CsCl gradient fractionation

For SIP200 and SIP2000 experiments, triplicate microcosms were sacrificed at day 5, 10 and 14. Soil samples (10 g) were transferred into 50-ml Falcon tubes and stored frozen at -80°C until use. Triplicate 10-g samples of untreated soil collected at the beginning of the experiment (day 0) served as controls. Duplicate soil samples were also collected at the endpoint (day 14) from control microcosms with no addition. DNA was extracted using the UltraClean Mega Soil DNA kit (MO BIO Laboratories) as recommended. DNA was eluted at 40-50 µg/ml in 8 mL.

For CsCl gradient fractionation, 2-ml portions of the DNA preparations were precipitated, and then adjusted to 700 ng/µl in H_2O. Separation between ^{12}C- and ^{13}C-DNA was performed by isopycnic ultracentrifugation on a CsCl gradient (Luederset al., 2004). Gradients were adjusted to an average density of 1.725 g/mL in 3.3 ml OptiSeal™ polyallomer centrifuge tubes (Beckman Coulter), and loaded with 30 to 50 micrograms of soil DNA and 5 µL of SYBR Safe™ (Invitrogen) as DNA stain (Martineauet al., 2008). For each run, a tube containing equal amounts of ^{12}C-DNA from *E. coli* and ^{13}C-DNA *Sphingomonas* sp. CHY-1 (10 µg each) was processed as a means to control band separation and locate their position in the gradient. ^{13}C-labeled genomic DNA was prepared from *Sphingomonas* sp. CHY-1 grown on ^{13}C-succinate (Sigma-Aldrich) as sole carbon source. Preparation of genomic DNA from this strain (Demanecheet al., 2004) and from *E. coli* strain DH5α (Ausubelet al., 1999) followed published procedures. Ultracentrifugation was carried out at 413,000 x g_{AV} for 17 h at 15°C, in a TLN-100 rotor using an Optima™ TLX Ultracentrifuge (Beckman Coulter). Gradient fractionation was adapted from a published procedure (Manefieldet al., 2002), using a peristaltic pump operated at a flow rate of 0.18 ml/min and a Gilson fraction collector equipped with a home-made device for holding centrifuge tubes. Twenty-two fractions, 150

192 µl each, were obtained, from which DNA was precipitated according to (Pumphrey and
193 Madsen, 2008), using 1 µl of 20 mg/ml glycogen as a carrier (Fermentas). Each fraction was
194 taken up in 20 µl H_2O and DNA concentration was determined from UV absorbance
195 measurement with a ND-100 spectrophotometer (NanoDrop Technologies, Inc.). In plots of
196 DNA content versus fraction number, peak fractions of ^{13}C-DNA and unlabeled DNA were 4
197 fractions apart.
198
199 **Construction of 16S rRNA gene libraries and DNA sequencing**
200 Both the labeled and unlabeled DNA isolated from the SIP200 and SIP2000 experiments, as
201 well as DNA extracted from untreated soil (control SIP 0D), was used as template to amplify
202 the nearly complete sequences of bacterial 16S rRNA genes. PCR amplification was
203 performed with primers 8F (AGAGTTTGATCCTGGCTCAG) and 1390R
204 (GACGGGCGGTGTGTACAA). PCR products were ligated into pCR™4 TOPO® TA
205 vectors and electroporated into ElectroMAX™ DH10B™ T1 competent cells according to the
206 manufacturer's recommendations (Invitrogen). Bidirectional Sanger sequence reads were
207 obtained by standard procedures and assembled by PHRAP (http://www.phrap.org). Sequence
208 data obtained from clone libraries have been submitted to DDBJ/EMBL/GenBank under
209 accession numbers: FQ658499 to FQ660546 and FQ790244.
210
211 **Sequence analysis**
212 The 16S rRNA gene sequences were first affiliated to b acterial taxa using SeqMatch on the
213 Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/index.jsp ; (Coleet al.,
214 2009)). Multiple sequence alignments and clustering into Operational Taxonomic Units
215 (OTUs) of the 2049 sequences considered herein were performed with mothur (Schlosset al.,
216 2009), using a 3% dissimilarity level between OTUs. Mothur was also used to generate

217 rarefaction curves and calculate richness estimators and diversity index (Table 1). A
218 phylogenetic tree was generated using the TreeBuilder software on the RDP website.
219
220 **Selection of 16S rRNA sequences representative of phenanthrene degraders**
221 In DNA-SIP analysis, the set of 16S RNA sequences recovered from ^{13}C-DNA may be
222 contaminated by unspecific sequences due to some overlap between labeled and unlabeled
223 fractions after isopycnic centrifugation. A control experiment is usually carried out involving
224 DNA analysis of a sample incubated with unlabeled substrate. After CsCl gradient separation,
225 a fraction equivalent to that containing ^{13}C-DNA in the labeled experiment (heavy fraction), is
226 chosen to run control PCR. In our hands, the choice of the heavy fraction for control purposes
227 was somewhat arbitrary due to small changes in gradient density between samples. In
228 addition, the reliability of this type of control requires that incubation conditions be identical
229 between the SIP and control samples, which is difficult to achieve due to the inherent
230 heterogeneity of soil. Hence, in his study, 16S rRNA sequences from ^{13}C-DNA libraries were
231 considered representative of phenanthrene degraders if they complied with the following
232 criteria : (i) they had to be part of OTUs represented by a minimum of 3 sequences per library
233 (ii) they were not detected or poorly represented in libraries made from unlabeled DNA from
234 the same run of centrifugation.
235
236 **Real time PCR quantification of phylum-specific 16S rRNA genes**
237 The copy number of 16S rRNA genes in soil DNA samples was estimated by quantitative
238 PCR (qPCR) using universal or taxon-specific primers according to (Philippotet al., 2010).
239 Amplification reactions were carried out in a StepOnePlus™ Real-Time PCR Systems
240 (Applied Biosystems). Reaction mixtures contained 7.5 µl SYBRGreen® PCR Master Mix
241 (Absolute QPCR SYBR Green Rox Abgene), 250 ng of T4 gene 32 (QBiogene), 4 ng of soil

242 DNA in a final volume of 15 µl. Fluorescence acquisition was performed during 80°C steps to
243 avoid interference of unspecific products. For each of the eleven 16S rRNA targets, a standard
244 curve was established using serial dilutions of linearized plasmid pGEM-T (10^2 to 10^7 copies)
245 containing a relevant 16S rRNA gene. Melting curves were generated after amplification by
246 increasing the temperature from 80°C to 95°C. qPCR results are averages of three replicates,
247 and are expressed as copy numbers per nanogram of DNA. The relative abundance of each
248 taxon was calculated as the ratio of copy number of this taxon to the total number of 16S
249 rRNA sequences, determined using universal primers.
250

RESULTS

Mineralization rates of ^{13}C-phenanthrene in microcosms

The soil used in this study had been exposed to chronic hydrocarbon contamination from road runoffs. It contained ca. 4.0 mg/kg of PAHs, including 0.156 ± 0.05 mg/kg of phenanthrene. For SIP experiments, fully labeled ^{13}C-phenanthrene was prepared and checked for purity and authenticity by GC-MS and NMR (supplementary Fig. S1). Experiments were carried out in microcosms containing 20 g of soil, and ^{13}C-phenanthrene supplied at 34 and 337 ppm, levels equivalent to approx. 200-fold (SIP200) or 2000-fold (SIP2000) the phenanthrene content of soil. Mineralization of the labeled C-source was monitored by GC-MS measurement of the $^{13}CO_2$ released in the headspace over 14 days (Fig. 1). In SIP2000 experiments, the $^{13}CO_2$ evolution reached a maximum rate after a 2-day lag phase, then leveled off after day 5. No lag phase was observed in microcosms inoculated with *Sphingomonas* CHY-1, a phenanthrene-degrading strain, suggesting that the delay reflected the time necessary for multiplication of soil PAH degraders. In SIP200 experiments, the rate of $^{13}CO_2$ was just above the background level detected in control microcosms, which received unlabelled phenanthrene or no addition. From the total amount of $^{13}CO_2$ released in the SIP2000 experiment, it was calculated that ca. 20% of the ^{13}C-phenanthrene had been mineralized. This value is most likely underestimated as an unknown amount of the carbon dioxide remained trapped in soil as bicarbonate. The residual labeled substrate in soil of the SIP2000 experiment amounted to 20.9 ± 2.3 ppm at day 5 (6.7 %) and 13.9 ± 1.0 ppm at day 14 (4.5 %), meaning that a major part of the added hydrocarbon had been degraded during the early stage of mineralization between days 2 and 5. Discrepancy between the mineralization rate and the extent of degradation of ^{13}C-phenanthrene (95%) might be explained in part by underestimations of either $^{13}CO_2$ (see above) or residual phenanthrene due to sequestration into soil particles (Johnsenet al., 2005).

Some of the labeled carbon was also incorporated into the organic matter of phenanthrene degraders.

Effect of phenanthrene on the overall bacterial community structure

To follow changes in the soil bacterial community upon incubation with phenanthrene, soil DNA was extracted at time intervals from SIP and control experiments, and 16S rRNA genes were first analyzed by SSCP fingerprinting. Electrophoresis profiles of PCR products targeting the V3 region were normalized and their distribution was analyzed by non metric multi-dimensional scaling (Fig. 2). Profiles were very similar suggesting that, at this level of observation, the bacterial community underwent little changes with the time of exposition (5, 10 or 14 d) and the dose of phenanthrene. Nevertheless, a clear trend emerged when comparing data sets at day 0, 5, 10 and 14, in that diversity profiles showed convergent time-dependent variations. On the other hand, diversity profiles obtained from phenanthrene-treated microcosms were more closely related to each other than to untreated controls, suggesting that phenanthrene-dependent shifts occurred in the bacterial population, but these shifts were little correlated to the concentration of phenanthrene.

The composition of the bacterial population in soil samples was then analyzed by real-time quantitative PCR (qPCR) using phylum-specific primers (class-specific for Proteobacteria), according to a method that has proven (Philippot, et al., 2010). Samples from the SIP2000 experiments showed, small time-dependent variations in 16S rRNA copy numbers (data not shown), suggesting that overall, the community structure was little affected by phenanthrene. However, when abundances were expressed as ratios with respect to the total copy numbers of 16S rRNA genes, a significant increase was found for the Beta- and Gammaproteobacteria (Fig. 3, grey bars). In contrast, Alphaproteobacteria and Actinobacteria showed unchanged or diminished proportions.

Betaproteobacteria enrichment in soil spiked with phenanthrene

Soil bacteria likely involved in phenanthrene degradation were identified based upon sequence analysis of 16S rRNA genes amplified from ^{13}C-DNA. Soil DNA samples recovered from SIP200 and SIP2000 experiments at day 5 and day 14 were separated into labeled and unlabeled fractions, both of which were used to prepare 16S rRNA gene libraries (Table 1). Sequences were affiliated to bacterial taxa using the RDP resources.. A detailed compilation of the 2049 sequences analyzed in this study is given in supplementary Table S1. As depicted in Fig. 4, the distribution of sequences among known bacterial phyla revealed that Proteobacteria were predominant in all libraries, the Beta class being the best represented. However, the proportion of Betaproteobacteria sequences was significantly higher in libraries made from labeled DNA, especially SIP2000 at day 5 (67%). In comparison, the library made out of unlabeled DNA (SIP2000 5D 12C) contained around 30% of Betaproteobacteria sequences, similar to the library of the untreated control (Fig. 4A and data not show). In the ^{13}C-DNA extracted from soil dosed with 10-fold less ^{13}C-phenanthrene, a Betaproteobacteria enrichment was also detectable, but only after 14 days (Fig. 4C).

Since the PCR-based method used to generate 16S rRNA sequence libraries might introduce biases in the determination of the actual proportions of bacterial taxa in soil, we implemented the quantitative PCR method described above to assess the copy number of taxon-specific16S rRNA genes in ^{13}C-DNA fractions. Results illustrated in Fig. 3 showed that labeled DNA was specifically enriched in sequences affiliated to the Betaproteobacteria. The enrichment occurred within the first days of the incubation with ^{13}C-phenanthrene, and the proportion of Betaproteobacteria stayed above 10% throughout the 14-d incubation. Gammaproteobacteria were found to be significantly more abundant at day 10, suggesting that members of this class also accumulated at least transiently in response to phenanthrene. In comparison, Alphaproteobacteria, and Actinobacteria, as well as other taxa tested (data not shown) were

326 less represented and their copy number did not show a clear trend upon incubation with
327 phenanthrene. Hence, consistent with the sequence analyses above, our quantitative data
328 demonstrated that Betaproteobacteria became the dominant taxon in response to phenanthrene
329 contamination of soil.
330
331 **Identification of main PAH degraders**
332 Further analysis of sequences in ^{13}C-libraries indicated that, at day 5, the community of PAH
333 degraders exposed to 337 ppm phenanthrene was dominated by a few genera, including
334 *Acidovorax, Rhodoferax, Hydrogenophaga* and *Polaromonas*, all members of the
335 Comamonadaceae (Fig. 5A). Quite a few sequences affiliated to Rhodocyclaceae were also
336 identified, but they were not all representative of phenanthrene degraders. Some of them were
337 likely related to degraders as they belonged to OTUs only found in ^{13}C-libraries (OTU0, 1
338 and 17), while other were not because they belonged to OTUs mainly found in ^{12}C-libraries
339 (OTU28, 51, 71; Fig. 6A). Hence, only some members of the Rhodocyclaceae would be able
340 to degrade phenanthrene. A very similar pattern of dominant taxa was observed in the SIP200
341 experiment at day 14 (Fig. 5B), indicating that a 10-fold lower concentration of phenanthrene
342 elicited the same soil population of degraders, although at a slower pace. Consistent with this
343 idea, comparison of the SIP2000-5D-13C and SIP200-14D-13C libraries showed that they
344 shared the highest number of common OTUs (78 of 161 or 48%, Fig S2), three of which were
345 dominant in both libraries (OTUs 6, 4 and 17; Fig. 6A).
346 Apart from the Betaproteobacteria, two OTUs related to the Sphingomonadales were
347 significantly represented in sequences obtained from ^{13}C-DNA, while almost inexistent in
348 sequences retrieved from unlabeled DNA (OTUs 80 and 201; Fig. 6A). Sphingomonadales
349 accounted for 32 to 37% of the Alphaproteobacteria sequences in SIP200 ^{13}C-libraries, and
350 for 38 to 59% in SIP2000 ^{13}C-libraries, respectively (Fig. S3). One well-represented OTU

351 related to unclassified Gammaproteobacteria (OTU 2, 5% of the SIP2000-5D-13C library)
352 was also detected in the set of sequences obtained from ^{13}C-DNA (Fig. 6A).
353 A phylogenetic tree was built with the best represented OTUs found in sequence libraries
354 derived from ^{13}C-DNA (Fig. 6B). The analysis further highlighted that phenanthrene
355 degraders were dominated by Betaproteobacteria. Except for OTU 4, OTU 6, OTU 12,
356 OTU 14 and OTU 27, which were closely related to known isolates, most sequences were
357 either associated to uncultured microorganisms or distantly related to known bacteria. In this
358 respect, OTU 80 and OTU 201, affiliated to Sphingomonadaceae, showed sequences
359 relatively distant from that of known PAH degraders in this bacterial family (Pinyakonget al.,
360 2003; Demaneche, et al., 2004).
361
362 **Diversity shifts in the PAH-degrading community as a function of the time of exposure**
363 **to phenanthrene**
364 Comparison of the SIP2000 sequence sets obtained from ^{13}C-DNA at 5 and 14 days showed
365 that the proportion of Betaproteobacteria dropped from 67 to 35% (Fig. 4D and E). This
366 change could largely be explained by a decline of the bacterial taxa that were identified as
367 dominant PAH degraders at day 5 (Fig. 5A). At day 14, the best-represented
368 Betaproteobacteria sequences were affiliated to *Thiobacillus*, a genus that has been seldom
369 described for its ability to degrade aromatic hydrocarbons although it was recognized as a
370 possible phenanthrene degrader (Bodouret al., 2003). A significant proportion of
371 *Thiobacillus*-like sequences was also detected in the SIP200 sequence sets retrieved from ^{13}C-
372 DNA. Similar to what was observed for the Betaproteobacteria, the OTU 2 related to the
373 Gammaproteobacteria was detected in the SIP2000 library at day 5, but not at day 14.

374 On the other hand, Sphingomonads were detectable under all conditions tested, but their
375 number tended to increase in the long run as exemplified by OTU 201, which was undetected
376 at day 5 and represented by 11 sequences (50% of Alphaproteobacteria) at day 14 (Fig. 6A).
377
378 **DISCUSSION**
379 PAH pollution is persistent in various ecosystems including soils all around the world, and
380 microbial biodegradation is considered the primary process responsible for its natural
381 attenuation. This process has been extensively studied but for a rather limited range of
382 culturable species (Peng, et al., 2008), and little is known on bacterial populations that mainly
383 contribute to PAH removal in situ. In the present study, we have implemented a SIP approach
384 and culture-independent methods to conduct a thorough investigation of phenanthrene
385 degraders present in soil.
386 Overall, the soil community was dominated by Proteobacteria, consistent with previous
387 reports documenting the prevalence of this phylum in different soils (Roeschet al., 2007).
388 Moreover, our results brought convergent pieces of evidence that Betaproteobacteria play a
389 major role in the degradation of phenanthrene in soil. This is supported by the increased
390 proportion of ribosomal sequences representative of this class in ^{13}C-DNA in response to
391 phenanthrene, as shown by both qPCR and sequence analysis of cloned 16S rRNA genes.
392 The main PAH degraders identified in this work mostly belong to the Burkholderiales, with
393 *Acidovorax, Rhodoferax, Hydrogenophaga* and *Polaromonas* as the best represented genera.
394 Likewise, a SIP study targeting naphthalene degraders, revealed that *Polaromonas,*
395 *Rhodoferax* and *Acidovorax* mainly contribute to in situ degradation (Jeon, et al., 2003),
396 suggesting that the same type of bacteria are responsible for the biodegradation of
397 naphthalene and phenanthrene in soil. Based on 16S rRNA sequence comparison, the
398 dominant *Acidovorax* identified in this study strikingly resembled members of the same genus

399 previously identified in a bioreactor treating a PAH-contaminated soil (Singleton, et al.,
400 2005). A strain isolated from this bioreactor, *Acidovorax* NA3, has been recently described as
401 a new PAH degrader (Singleton, et al., 2009).
402 Interestingly, most PAH degraders identified in the present study are related to poorly
403 described taxa, consistent with the fact that a majority of soil bacteria are unknown. They
404 differ from PAH-degrading isolates studied so far, and even members of well-known
405 degraders such as Sphingomonadaceae (Demaneche, et al., 2004; Leys, et al., 2004), were
406 found to be distantly related to described strains (Fig. 6). Gammaproteobacteria are
407 represented by one OTU unrelated to known genera of this class, such as *Pseudomonas*
408 (OTU2; Fig. 6), but very similar to a pyrene-degrading uncultured bacterium detected by SIP
409 in a bioreactor (Singleton, et al., 2006). Moreover, our survey highlighted bacterial taxa
410 affiliated to Rhodocyclaceae and *Thiobacillus,* which appear as new PAH degraders. Among
411 Rhodocyclaceae, denitrifying bacteria belonging to the *Azoarcus, Aromatoleum,*
412 *Denitratisoma and Thauera* genera were shown to anaerobically degrade phenolic
413 compounds (Sueokaet al., 2009) and alkylbenzenes (Rabus and Widdel, 1995; Rotaruet al.,
414 2010), but no Rhodocyclaceae member endowed with PAH-degrading ability has yet been
415 described.
416 Similar populations of degraders were found upon soil treatment with 10-fold different levels
417 of phenanthrene. This observation indicated that, at the highest concentration tested, no
418 adverse effect could be noticed on any of the bacterial taxa involved in phenanthrene
419 degradation. In contrast, different naphthalene degraders were identified in a ground aquifer,
420 depending on the concentration of the hydrocarbon (Huanget al., 2009). In the latter case, the
421 toxic or inhibitory effect of naphthalene might have influenced bacterial selection, in
422 accordance with previous observations (Jeon, et al., 2003).

Time-dependent changes in the population of degraders were observed upon incubation with phenanthrene. The rapid phase of phenanthrene mineralization during the first days coincided with the multiplication in soil of the main degraders discussed above. In the SIP2000 experiment, dominant degraders at day 5 drastically declined at day 14. During this time period, the soil bacterial community underwent little change as shown by SSCP and qPCR analyses. Hence, the decline appeared to specifically affect the subpopulation of PAH degraders, maybe because phenanthrene became less bioavailable. Although significant amounts of residual phenanthrene were detected in microcosms after 5 and 14 days, the time course of mineralization suggested that it became limiting at day 5, perhaps due to its sequestration or sorption on soil particles. In the SIP200 experiment, the lower dose of phenanthrene likely elicited slower growth, and might have sustained PAH degraders for a longer time. Hence, our results provide evidence that the subpopulation of degraders undergo relatively rapid dynamic changes in response to the level of PAHs available in soil.

Acknowledgments

We thank N. Duraffourg for assistance in recording RMN spectra, A. Monier and J. Roy for their help in SSCP experiments, E. Pelletier for submitting DNA sequences. F. Martin received a grant from the Rhône-Alpes region. This work was supported by the CNRS and the University of Grenoble.

REFERENCES

Alonso-Gutierrez, J., Figueras, A., Albaiges, J., Jimenez, N., Vinas, M., Solanas, A.M., Novoa, B., 2009. Bacterial communities from shoreline environments (Costa da Morte,

447 Northwestern Spain) affected by the Prestige oil spill. Appl Environ Microbiol 75, 3407-
448 3418.
449 Ausubel, F.M., Brent, R., Kingston, R., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K.,
450 1999. Short protocols in molecular biology. Academic press.
451 Bodour, A.A., Wang, J.-M., Brusseau, M.L., Maier, R.M., 2003. Temporal change in
452 culturable phenanthrene degraders in response to long-term exposure to phenanthrene in a soil
453 column system. Environ Microbiol 5, 888-895.
454 Cebron, A., Louvel, B., Faure, P., France-Lanord, C., Chen, Y., Murrell, J.C., Leyval, C.,
455 2011. Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted
456 soil but not phenanthrene degradation rates. Environ Microbiol 13, 722-736.
457 Cole, J.R., Konstantinidis, K., Farris, R.J., Tiedje, J.M., 2010. Microbial diversity and
458 phylogeny: extending from rRNAs to genomes, in: Liu, W.T., Jansson, J.K. (Eds.),
459 Environmental Molecular Microbiology. Caister Academic Press, pp. 1-19.
460 Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen,
461 A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal
462 Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res
463 37, D141-D145.
464 Demaneche, S., Meyer, C., Micoud, J., Louwagie, M., Willison, J.C., Jouanneau, Y., 2004.
465 Identification and functional analysis of two aromatic-ring-hydroxylating dioxygenases from
466 a *Sphingomonas* strain that degrades various polycyclic aromatic hydrocarbons. Appl Environ
467 Microbiol 70, 6714-6725.
468 Doyle, E., Muckian, L., Hickey, A.M., Clipson, N., 2008. Microbial PAH degradation. Adv.
469 Appl. Microbiol. 65, 27-66.
470 Dumont, M.G., Murrell, J.C., 2005. Stable isotope probing - linking microbial identity to
471 function. Nat Rev Microbiol 3, 499-504.

472 Huang, W.E., Ferguson, A., Singer, A.C., Lawson, K., Thompson, I.P., Kalin, R.M., Larkin,
473 M.J., Bailey, M.J., Whiteley, A.S., 2009. Resolving genetic functions within microbial
474 populations: In situ analyses using rRNA and mRNA stable isotope probing coupled with
475 single-cell Raman-fluorescence in situ hybridization. Appl Environ Microbiol 75, 234-241.
476 Jeon, C.O., Park, W., Padmanabhan, P., DeRito, C.J., Snape, R., Madsen, E.L., 2003.
477 Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ
478 biodegradation in contaminated sediment. Proc Natl Acad Sci USA 100 13591-13596.
479 Johnsen, A.R., Wick, L.Y., Harms, H., 2005. Principles of microbial PAH-degradation in soil.
480 Environ. Pollut. 133, 71-84.
481 Jones, M.D., Singleton, D.R., Sun, W., Aitken, M.D., 2011. Multiple DNA extractions
482 coupled with stable-isotope probing of anthracene-degrading bacteria in contaminated soil.
483 Appl. Environ. Microbiol. 77, 2984-2991.
484 Krivobok, S., Kuony, S., Meyer, C., Louwagie, M., Willison, J.C., Jouanneau, Y., 2003.
485 Identification of pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1: evidence for two
486 ring-hydroxylating dioxygenases. J Bacteriol 185, 3828-3841.
487 Leys, N.M., Ryngaert, A., Bastiaens, L., Wattiau, P., Top, E.M., Verstraete, W., Springael,
488 D., 2005. Occurrence and community composition of fast-growing *Mycobacterium* in soils
489 contaminated with polycyclic aromatic hydrocarbons. FEMS Microbiol Ecol 51, 375-388.
490 Leys, N.M.E.J., Ryngaert, A., Bastiaens, L., Verstraete, W., Top, E.M., Springael, D., 2004.
491 Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with
492 polycyclic aromatic hydrocarbons. Appl Environ Microbiol 70, 1944-1955.
493 Lueders, T., Manefield, M., Friedrich, M.W., 2004. Enhanced sensitivity of DNA- and rRNA-
494 based stable isotope probing by fractionation and quantitative analysis of isopycnic
495 centrifugation gradients. Environ Microbiol 6, 73-78.

496 Manefield, M., Whiteley, A.S., Griffiths, R.I., Bailey, M.J., 2002. RNA stable isotope
497 probing, a novel means of linking microbial community function to Phylogeny. Appl Environ
498 Microbiol 68, 5367-5373.
499 Martineau, C., Whyte, L.G., Greer, C.W., 2008. Development of a SYBR safe (TM)
500 technique for the sensitive detection of DNA in cesium chloride density gradients for stable
501 isotope probing assays. J Microbiol Methods 73, 199-202.
502 Peng, R.H., Xiong, A.S., Xue, Y., Fu, X.Y., Gao, F., Zhao, W., Tian, Y.S., Yao, Q.H., 2008.
503 Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Rev 32, 927-955.
504 Philippot, L., Tscherko, D., Bru, D., Kandeler, E., 2010. Distribution of high bacterial taxa
505 across the chronosequence of two alpine glacier forelands. Microb Ecol 61, 303-312.
506 Pinyakong, O., Habe, H., Omori, T., 2003. The unique aromatic catabolic genes in
507 sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs). J Gen Appl Microbiol
508 49, 1-19.
509 Pumphrey, G.M., Madsen, E.L., 2008. Field-based stable isotope probing reveals the
510 identities of benzoic acid-metabolizing microorganisms and their in situ growth in agricultural
511 soil. Appl Environ Microbiol 74, 4111-4118.
512 Rabus, R., Widdel, F., 1995. Anaerobic degradation of ethylbenzene and other aromatic-
513 hydrocarbons by new denitrifying bacteria. Arch Microbiol 163, 96-103.
514 Radajewski, S., Ineson, P., Parekh, N.R., Murrell, J.C., 2000. Stable-isotope probing as a tool
515 in microbial ecology. Nature 403, 646-649.
516 Roesch, L.F.W., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K.M., Kent, A.D., Daroub,
517 S.H., Camargo, F.A.O., Farmerie, W.G., Triplett, E.W., 2007. Pyrosequencing enumerates
518 and contrasts soil microbial diversity. ISME J 1, 283-290.
519 Rotaru, A.-E., Probian, C., Wilkes, H., Harder, J., 2010. Highly enriched Betaproteobacteria
520 growing anaerobically with p-xylene and nitrate. FEMS Microbiol Ecol 71, 460-468.

521 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B.,
522 Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger,
523 G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-
524 independent, community-supported software for describing and comparing microbial
525 communities. Appl. Environ. Microbiol. 75, 7537-7541.
526 Singleton, D.R., Powell, S.N., Sangaiah, R., Gold, A., Ball, L.M., Aitken, M.D., 2005. Stable-
527 isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a
528 bioreactor treating contaminated soil. Appl Environ Microbiol 71, 1202-1209.
529 Singleton, D.R., Ramirez, L.G., Aitken, M.D., 2009. Characterization of a polycyclic
530 aromatic hydrocarbon degradation gene cluster in a phenanthrene-degrading *Acidovorax*
531 strain. Appl Environ Microbiol 75, 2613-2620.
532 Singleton, D.R., Sangaiah, R., Gold, A., Ball, L.M., Aitken, M.D., 2006. Identification and
533 quantification of uncultivated proteobacteria associated with pyrene degradation in a
534 bioreactor treating PAH-contaminated soil. Environ Microbiol 8, 1736-1745.
535 Sueoka, K., Satoh, H., Onuki, M., Mino, T., 2009. Microorganisms involved in anaerobic
536 phenol degradation in the treatment of synthetic coke-oven wastewater detected by RNA
537 stable-isotope probing. FEMS Microbiol Lett 291, 169-174.
538 Wackett, L.P., 2004. Stable isotope probing in biodegradation research. Trends biotechnol.
539 22, 153-154.
540 Willison, J.C., 2004. Isolation and characterization of a novel sphingomonad capable of
541 growth with chrysene as sole carbon and energy source. FEMS Microbiol Lett 241, 143-150.
542 Zinger, L., Gury, J., Giraud, F., Krivobok, S., Gielly, L., Taberlet, P., Geremia, R.A., 2007.
543 Improvements of polymerase chain reaction and capillary electrophoresis single-strand
544 conformation polymorphism methods in microbial ecology: Toward a high-throughput
545 method for microbial diversity studies in soil. Microb Ecol 54, 203-216.

546 **Table 1 : Description of the nine 16S rRNA sequence libraries considered in the DNA SIP experiments.**

547

Library	Sequence number	OTU number	Richness estimation		Diversity Index
			Chao1	Ace	Shannon
SIP1 0D	230	173	468	892	4.87
SIP200 5D 13C	167	132	810	783	4.67
SIP200 5D 12C	147	135	938	1897	4.82
SIP200 14D 13C	353	161	748	2144	4.32
SIP200 14D 12C	259	233	572	956	4.96
SIP2000 5D 13C	423	173	536	942	3.89
SIP2000 5D 12C	228	186	739	1981	5.01
SIP2000 14D 13C	212	135	476	877	4.20
SIP2000 14D 12C	28	27	118	182	3.23

548 **Legends to figures**

549

550 Figure 1: Time course of $^{13}CO_2$ evolution during SIP experiments as measured by GC/MS.
551 $^{13}CO_2$ was measured in the headspace of microcosms supplied with either 34 ppm (△;
552 SIP200) or 337 ppm (▲; SIP2000) ^{13}C-phenanthrene, at the beginning of the incubation. Two
553 microcosms spiked with 337 ppm ^{13}C-phenanthrene were inoculated with *Sphingomonas*
554 CHY-1 (◆). Other experimental conditions included addition of 34 ppm unlabelled
555 phenanthrene (□), no addition (■) and abiotic control (○). Data show net $^{13}CO_2$ evolution
556 calculated by subtracting natural $^{13}CO_2$ present in the air. Data are averages of three replicate
557 treatments for SIP200 and SIP2000, and two replicates for other experimental conditions.
558 Error bars indicate standard deviations.

559

560 Figure 2: NMDS ordination of bacterial communities exposed to phenanthrene, based on
561 SSCP fingerprint analysis.
562 SSCP profiles were computed and compared through nonmetric multidimensional scaling
563 using a stress value of 9.46. Data fitting was applied to examine the effects of phenanthrene
564 and incubation time on the ordination. Phenanthrene concentration was 34 ppm (■), 337 ppm
565 (▲) or background level (●). Incubation time (days) was 0 (orange symbols), 5 (red), 10
566 (blue), or 14 (green). Vector indicates time variable ($P < 0.001$), centroides denote
567 phenanthrene level added: none (PheNo), 34 ppm (PheLw) or 337 ppm (PheHg) ($P < 0.002$).

568

569 Figure 3: Relative abundances of phylum-specific 16S rRNA genes in soil DNA during
570 incubation with ^{13}C-phenanthrene. Template was either total soil DNA (grey bars) or ^{13}C-
571 DNA (black bars) extracted from SIP2000 experiments at 5, 10 and 14 days (5D, 10D and
572 14D, respectively). A control DNA sample was obtained from soil incubated for 14 days

573 without phenanthrene (T14D). Stars denote significant differences compared to control DNA
574 from non-incubated untreatz)ed soil (0D ; *P<0.05*). A: Alphaproteobacteria; B:
575 Betaproteobacteria; C: Gammaproteobacteria; D: Actinobacteria.
576
577 Figure 4: Prevalence of Betaproteobacteria-specific sequences in 16S rRNA gene libraries
578 recovered from ^{13}C-labeled soil DNA.
579 Chart pies represent sequence repartition into main taxa for the following 16S rRNA gene
580 libraries: A, SIP1 0D (control); B, SIP200 5D 13C; C, SIP200 14D 13C; D, SIP2000 5D 13C;
581 E, SIP2000 14D 13C. Other phyla include Bacteroidetes, Chloroflexi, Spirochete,
582 Verrucomicrobia, Cyanobacteria, Gemmatimonadetes, Firmicutes, Planctomycete and
583 Nirospira.
584
585 Figure 5 : Distribution of Betaproteobacteria into best-represented families and genera.
586 Sequences were from ^{13}C-DNA libraries prepared at day 5 and 14 from the SIP2000 (A) and
587 the SIP200 (B) experiments, or from a control DNA library made from untreated soil (SIP
588 0D). Ratios were calculated as the number of sequences per taxon versus the total number of
589 sequences affiliated to Betaproteobacteria in each library.
590
591 Figure 6: Major bacterial taxa likely involved in ^{13}C-phenanthrene degradation.
592 From a compilation of all 2049 16S rRNA gene sequences considered herein, grouping in
593 OTUs was performed with mothur. A : Diagram showing the sequence number of those
594 OTUs exclusively or mainly consisting of sequences derived from ^{13}C-DNA. B : Phylogenetic
595 tree illustrating the relationships between these OTUs and most similar sequences found in
596 the RDP data-base. Affiliation to relevant bacterial taxa is indicated on the left. The tree was

built with TreeBuilder found on the RDP website, using the *Aquifex pyrophilus* 16S rRNA gene as an outgroup sequence.

Figure
Click here to download Figure: Figure 1.pdf

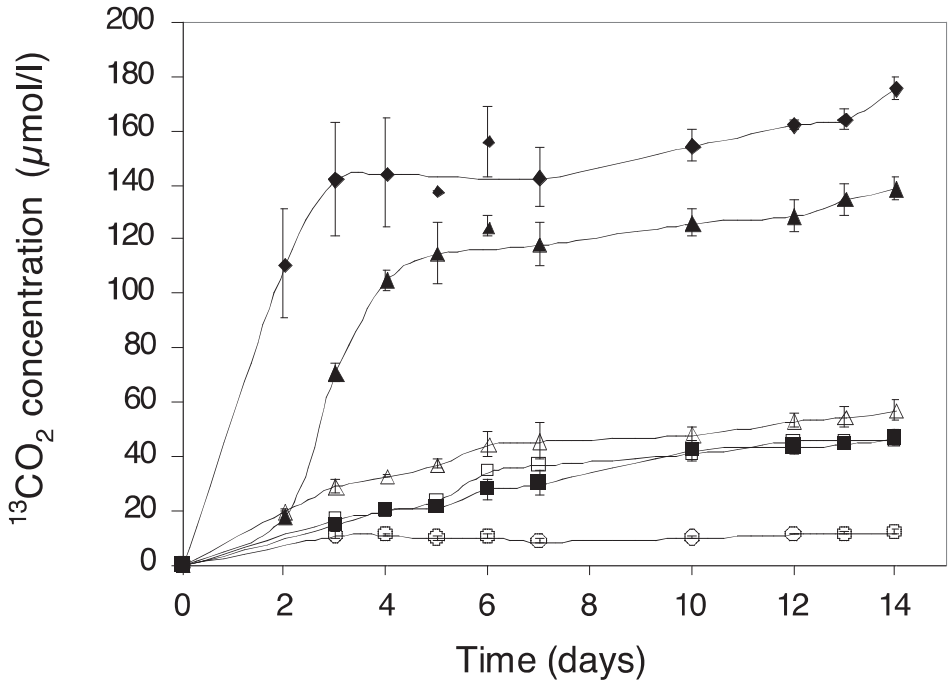


Figure 1: Time course of $^{13}CO_2$ evolution during SIP experiments as measured by GC/MS. $^{13}CO_2$ was measured in the headspace of microcosms supplied with either 34 ppm (△; SIP200) or 337 ppm (▲; SIP2000) ^{13}C-phenanthrene, at the beginning of the incubation. Two microcosms spiked with 337 ppm ^{13}C-phenanthrene were inoculated with *Sphingomonas* CHY-1 (◆). Other experimental conditions included addition of 34 ppm unlabelled phenanthrene (□), no addition (■) and abiotic control (○). Data show net $^{13}CO_2$ evolution calculated by subtracting natural $^{13}CO_2$ present in the air. Data are averages of three replicate treatments for SIP200 and SIP2000, and two replicates for other experimental conditions. Error bars indicate standard deviations.

Figure
Click here to download Figure: Figure 2.pdf

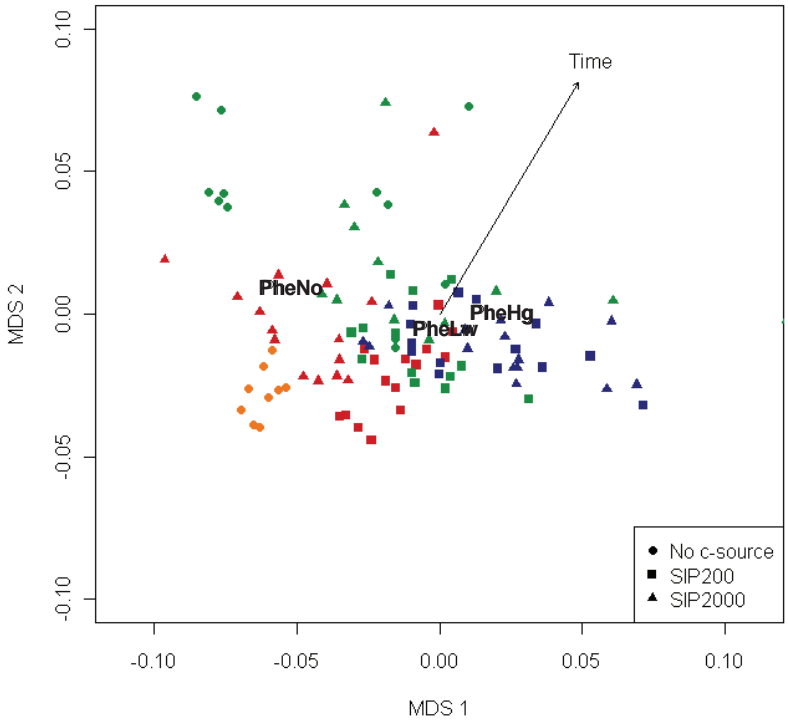


Figure 2: NMDS ordination of bacterial communities exposed to phenanthrene, based on SSCP fingerprint analysis.

SSCP profiles were computed and compared through non metric multidimensional scaling using a stress value of 9.46. Data fitting was applied to examine the effects of phenanthrene and incubation time on the ordination. Phenanthrene concentration was 31 ppm (■), 310 ppm (▲) or background level (●). Incubation time (days) was 0 (orange symbols), 5 (red), 10 (blue), or 14 (green). Vector indicates Time variable ($P < 0.001$), centroides denote phenanthrene level added: none (PheNo), 31 ppm (PheLw) or 310 ppm (PheHg) ($P < 0.002$).

Figure
Click here to download Figure: Figure 3.pdf

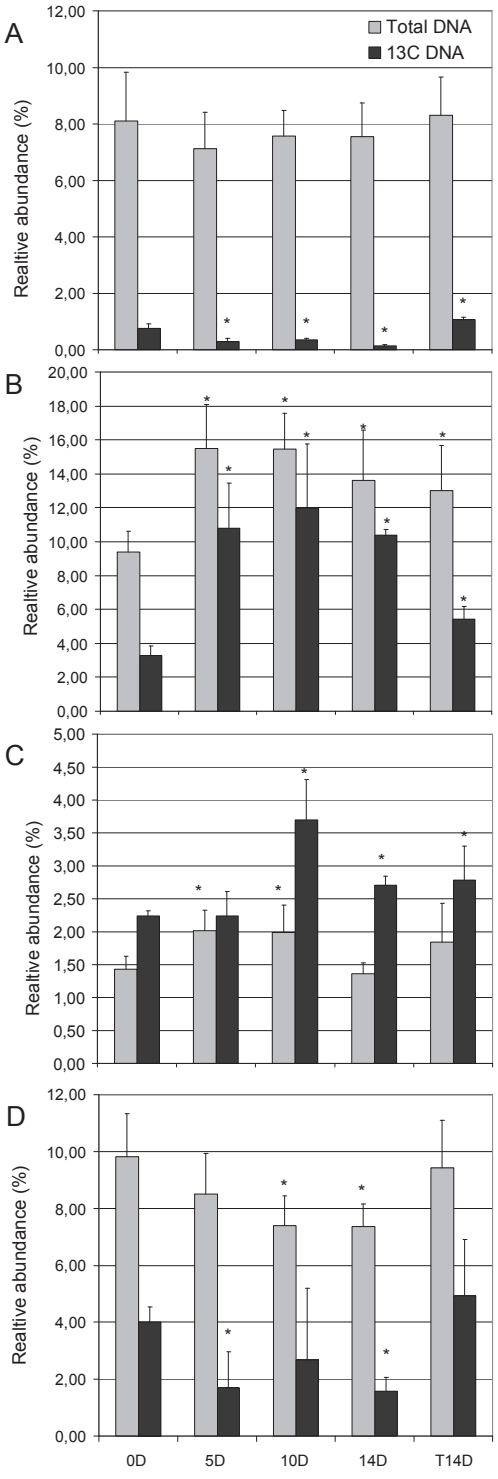


Figure 3: Relative abundances of phylum-specific 16S rRNA genes in soil DNA during incubation with ^{13}C-phenanthrene. Template was either total soil DNA (grey bars) or ^{13}C-DNA (black bars) extracted from SIP2000 experiments at 5, 10 and 14 days (5D, 10D and 14D, respectively). A control DNA sample was obtained from soil incubated for 14 days

without phenanthrene (T14D). Stars denote significant differences compared to control DNA from non-incubated soil (0D ; $p<0.05$). A: Alphaproteobacteria; B: Betaproteobacteria; C: Gammaproteobacteria; D: Actinobacteria.

Figure
Click here to download Figure: Figure 4.pdf

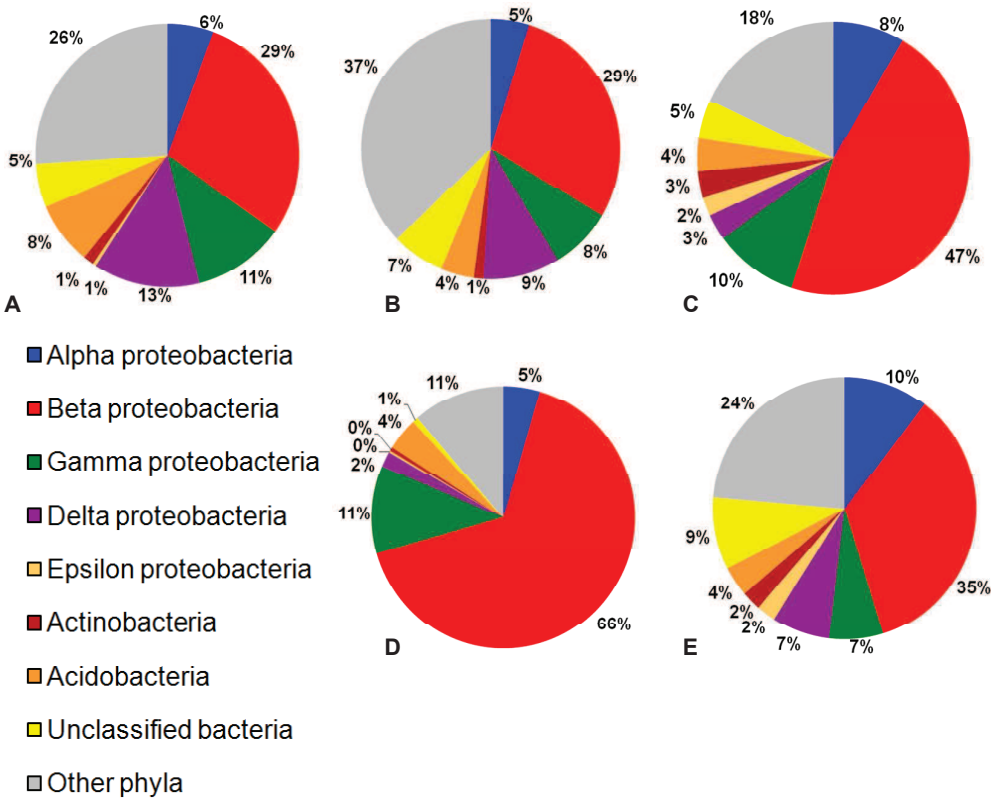


Figure 4: Prevalence of Betaproteobacteria-specific sequences in 16S rRNA gene libraries recovered from ^{13}C-labeled soil DNA.

Chart pies represent sequence repartition into main taxa for the following 16S rRNA gene libraries: A, SIP1 0D (control); B, SIP200 5D 13C; C, SIP200 14D 13C; D, SIP2000 5D 13C; E, SIP2000 14D 13C. "Other phyla" include *Bacteroidetes, Chloroflexi, Spirochete, Verrucomicrobia, Cyanobacteria, Gemmatimonadetes, Firmicutes, Planctomycete and Nirospira*.

Figure
Click here to download Figure: Figure 5.pdf

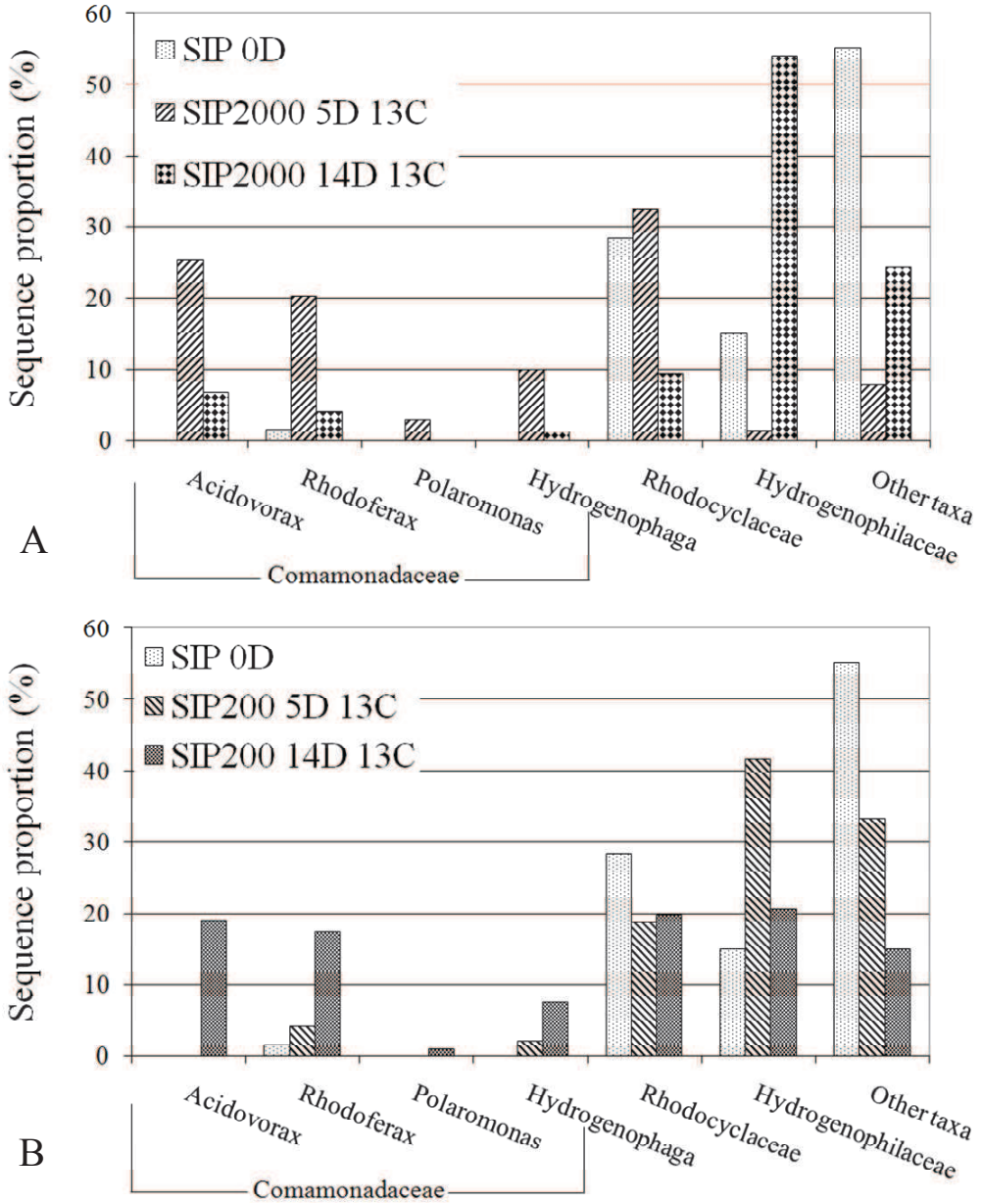
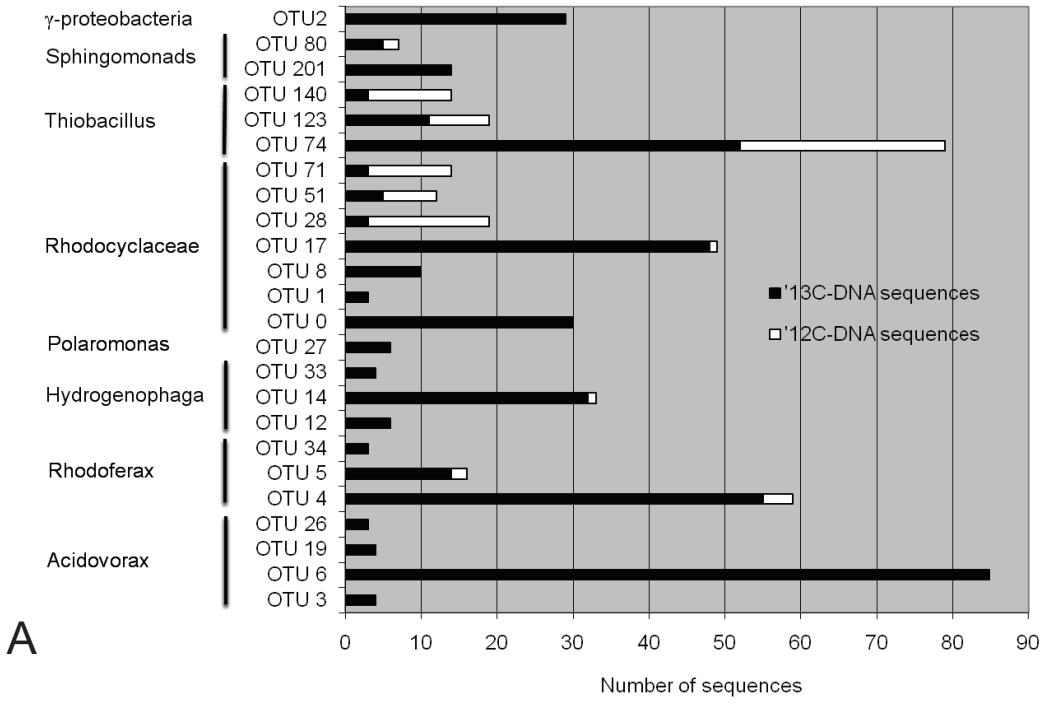
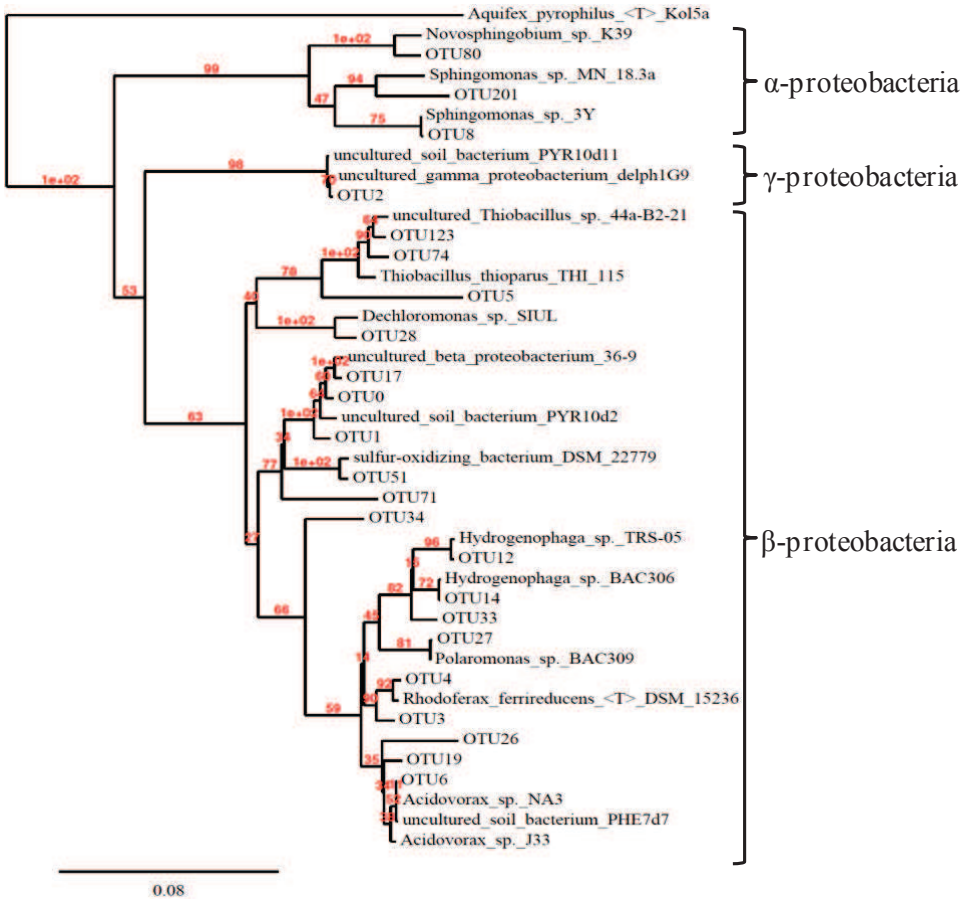


Figure 5: Distribution of Betaproteobacteria into best-represented families and genera. Sequences were from ^{13}C-DNA libraries of the SIP2000 (A) and the SIP200 (B) experiments, or from the untreated soil DNA library (SIP 0D). Ratios were calculated as the number of sequences per taxon versus the total number of sequences affiliated to Betaproteobacteria in each library.

Figure
Click here to download Figure: Figure 6.pdf



A



B

Figure 6

Figure 6 : Major bacterial taxa likely involved in ^{13}C-phenanthrene degradation.

From a compilation of all 2049 16S rRNA gene sequences considered herein, grouping in OTUs was performed with mothur. A : Diagram showing the sequence number of those OTUs exclusively or mainly consisting of sequences derived from ^{13}C-DNA. B : Phylogenetic tree illustrating the relationships between these OTUs and most similar sequences found in the RDP data-base. Affiliation to relevant bacterial taxa is indicated on the left. The tree was built with TreeBuilder found on the RDP website, using the *Aquifex pyrophilus* 16S rRNA gene as an outgroup sequence.

Figure 6

Supplementary Files

Click here to download Supplementary Files: Table S1.xls

Supplementary Files

Click here to download Supplementary Files: Figures S1-S3.pdf