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

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2   **PAH-contaminated soil exposed to phenanthrene**

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25   **Abstract**

26   In this study, the PAH-degrading bacteria of a constructed wetland collecting road runoff has

27   been studied through DNA stable isotope probing. Microcosms were spiked with ^{13}C-

28   phenanthrene at 34 or 337 ppm, and bacterial diversity was monitored over a 14-day period.

29   At 337 ppm, PAH degraders became dominated after 5 days by Betaproteobacteria, including

30   novel *Acidovorax*, *Rhodoferax* and *Hydrogenophaga* members, and unknown bacteria related

31   to Rhodocyclaceae. The prevalence of Betaproteobacteria was further demonstrated by

32   phylum-specific quantitative PCR, and was correlated with a burst of phenanthrene

33   mineralization. Striking shifts in the population of degraders were observed after most of the

34   phenanthrene had been removed. Soil exposed to 34 ppm phenanthrene showed a similar

35   population of degraders, albeit only after 14 days. Our results indicate that specific

36   Betaproteobacteria are involved in the main response to soil PAH contamination, and

37   illustrate the potential of SIP approaches to identify soil PAH degraders.

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

39   Betaproteobacteria

40

41

42   Capsule : On a site collecting road runoff, implementation of stable isotope probing to

43   identify soil bacteria responsible for phenanthrene degradation, led to the discovery of new

44   Betaproteobacteria distantly related to known PAH degraders.

45

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$_4$)$_2$SO$_4$ – 20 mM KH$_2$PO$_4$ – 30.6 mM Na$_2$HPO$_4$ – 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

117   2000-fold as high as that of phenanthrene initially found in soil. Relevant microcosm

118   experiments were referred to as SIP200 and SIP2000, respectively. Two microcosms supplied

119   with 337 ppm labeled phenanthrene were inoculated with approx. 2 10^8 cells (0.2 ml) of a

120   washed culture of *Sphingomonas* CHY-1 (Willison, 2004). Three types of control flasks were

121   also prepared, which contained either unlabeled phenanthrene (added at 34 ppm as for

122   SIP200), no additional carbon source, or sodium azide (7.7 10^{-5} mol/g dry soil; abiotic

123   control). Microcosms containing [U-^{13}C]-phenanthrene (SIP200 and SIP2000) were prepared

124   in 9 replicates (triplicates at 3 time points), whereas controls with unlabelled phenanthrene or

125   microcosms inoculated with strain CHY-1 were performed in 6 replicates (duplicates at 3

126   time points). Other control flasks were incubated in duplicates. Flasks were incubated at 25°C

127   in static mode in a dark room.

128

129   **PAH extraction from soil and quantification**

130   Soil samples (1g) were mixed with 1g anhydrous sodium sulfate and 10 mL hexane in 30-mL

131   glass tubes closed with Teflon-sealed screw-caps. Suspensions were homogenized by Vortex

132   mixing and treatment in an ultrasonic bath for a total time of 1 h. Soil samples were extracted

133   once more with hexane and organic phases were combined, centrifuged for 12 min at 7400 *g*

134   before evaporation to dryness under argon. Dry extracts were dissolved in 0.5 ml acetonitrile.

135   Residual ^{13}C-phenanthrene was extracted in the same way.

136   Quantification of the 16 priority PAHs was performed with a HP6890 gas chromatograph

137   coupled to a HP5973 mass spectrometer (Agilent Technologies). PAHs were separated on a

138   MDN12 column (30 m by 0.25 mm, 25µm film thickness; Supelco) as previously described

139   (Krivoboket al., 2003). The mass detector was operated in the single-ion monitoring mode.

140   PAHs were eluted between 8.9 and 32.7 min. Concentrations were determined using

141   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.

168   **DNA extraction and CsCl gradient fractionation**

169   For SIP200 and SIP2000 experiments, triplicate microcosms were sacrificed at day 5, 10 and

170   14. Soil samples (10 g) were transferred into 50-ml Falcon tubes and stored frozen at -80°C

171   until use. Triplicate 10-g samples of untreated soil collected at the beginning of the

172   experiment (day 0) served as controls. Duplicate soil samples were also collected at the

173   endpoint (day 14) from control microcosms with no addition. DNA was extracted using the

174   UltraClean Mega Soil DNA kit (MO BIO Laboratories) as recommended. DNA was eluted at

175   40-50 µg/ml in 8 mL.

176   For CsCl gradient fractionation, 2-ml portions of the DNA preparations were precipitated,

177   and then adjusted to 700 ng/µl in H_2O. Separation between ^{12}C- and ^{13}C-DNA was performed

178   by isopycnic ultracentrifugation on a CsCl gradient (Lueder et al., 2004). Gradients were

179   adjusted to an average density of 1.725 g/mL in 3.3 ml OptiSeal^{TM} polyallomer centrifuge

180   tubes (Beckman Coulter), and loaded with 30 to 50 micrograms of soil DNA and 5 µL of

181   SYBR Safe^{TM} (Invitrogen) as DNA stain (Martineau et al., 2008). For each run, a tube

182   containing equal amounts of ^{12}C-DNA from *E. coli* and ^{13}C-DNA *Sphingomonas* sp. CHY-1

183   (10 µg each) was processed as a means to control band separation and locate their position in

184   the gradient. ^{13}C-labeled genomic DNA was prepared from *Sphingomonas* sp. CHY-1 grown

185   on ^{13}C-succinate (Sigma-Aldrich) as sole carbon source. Preparation of genomic DNA from

186   this strain (Demaneche et al., 2004) and from *E. coli* strain DH5α (Ausubel et al., 1999)

187   followed published procedures. Ultracentrifugation was carried out at 413,000 x g_{AV} for 17 h

188   at 15°C, in a TLN-100 rotor using an Optima^{TM} TLX Ultracentrifuge (Beckman Coulter).

189   Gradient fractionation was adapted from a published procedure (Manefield et al., 2002), using

190   a peristaltic pump operated at a flow rate of 0.18 ml/min and a Gilson fraction collector

191   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

251 **RESULTS**

252

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

254 The soil used in this study had been exposed to chronic hydrocarbon contamination from road

255 runoffs. It contained ca. 4.0 mg/kg of PAHs, including 0.156 ± 0.05 mg/kg of phenanthrene.

256 For SIP experiments, fully labeled ^{13}C-phenanthrene was prepared and checked for purity and

257 authenticity by GC-MS and NMR (supplementary Fig. S1). Experiments were carried out in

258 microcosms containing 20 g of soil, and ^{13}C-phenanthrene supplied at 34 and 337 ppm, levels

259 equivalent to approx. 200-fold (SIP200) or 2000-fold (SIP2000) the phenanthrene content of

260 soil. Mineralization of the labeled C-source was monitored by GC-MS measurement of the

261 $^{13}CO_2$ released in the headspace over 14 days (Fig. 1). In SIP2000 experiments, the $^{13}CO_2$

262 evolution reached a maximum rate after a 2-day lag phase, then leveled off after day 5. No lag

263 phase was observed in microcosms inoculated with *Sphingomonas* CHY-1, a phenanthrene-

264 degrading strain, suggesting that the delay reflected the time necessary for multiplication of

265 soil PAH degraders. In SIP200 experiments, the rate of $^{13}CO_2$ was just above the background

266 level detected in control microcosms, which received unlabelled phenanthrene or no addition.

267 From the total amount of $^{13}CO_2$ released in the SIP2000 experiment, it was calculated that ca.

268 20% of the ^{13}C-phenanthrene had been mineralized. This value is most likely underestimated

269 as an unknown amount of the carbon dioxide remained trapped in soil as bicarbonate. The

270 residual labeled substrate in soil of the SIP2000 experiment amounted to 20.9 ± 2.3 ppm at

271 day 5 (6.7 %) and 13.9 ± 1.0 ppm at day 14 (4.5 %), meaning that a major part of the added

272 hydrocarbon had been degraded during the early stage of mineralization between days 2 and

273 5. Discrepancy between the mineralization rate and the extent of degradation of ^{13}C-

274 phenanthrene (95%) might be explained in part by underestimations of either $^{13}CO_2$ (see

275 above) or residual phenanthrene due to sequestration into soil particles (Johnsenet al., 2005).

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

277   degraders.

278   **Effect of phenanthrene on the overall bacterial community structure**

279   To follow changes in the soil bacterial community upon incubation with phenanthrene, soil

280   DNA was extracted at time intervals from SIP and control experiments, and 16S rRNA genes

281   were first analyzed by SSCP fingerprinting. Electrophoresis profiles of PCR products

282   targeting the V3 region were normalized and their distribution was analyzed by non metric

283   multi-dimensional scaling (Fig. 2). Profiles were very similar suggesting that, at this level of

284   observation, the bacterial community underwent little changes with the time of exposition (5,

285   10 or 14 d) and the dose of phenanthrene. Nevertheless, a clear trend emerged when

286   comparing data sets at day 0, 5, 10 and 14, in that diversity profiles showed convergent time-

287   dependent variations. On the other hand, diversity profiles obtained from phenanthrene-

288   treated microcosms were more closely related to each other than to untreated controls,

289   suggesting that phenanthrene-dependent shifts occurred in the bacterial population, but these

290   shifts were little correlated to the concentration of phenanthrene.

291   The composition of the bacterial population in soil samples was then analyzed by real-time

292   quantitative PCR (qPCR) using phylum-specific primers (class-specific for Proteobacteria),

293   according to a method that has proven (Philippot, et al., 2010). Samples from the SIP2000

294   experiments showed, small time-dependent variations in 16S rRNA copy numbers (data not

295   shown), suggesting that overall, the community structure was little affected by phenanthrene.

296   However, when abundances were expressed as ratios with respect to the total copy numbers

297   of 16S rRNA genes, a significant increase was found for the Beta- and Gammaproteobacteria

298   (Fig. 3, grey bars). In contrast, Alphaproteobacteria and Actinobacteria showed unchanged or

299   diminished proportions.

300

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

15

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

17

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).

423   Time-dependent changes in the population of degraders were observed upon incubation with

424   phenanthrene. The rapid phase of phenanthrene mineralization during the first days coincided

425   with the multiplication in soil of the main degraders discussed above. In the SIP2000

426   experiment, dominant degraders at day 5 drastically declined at day 14. During this time

427   period, the soil bacterial community underwent little change as shown by SSCP and qPCR

428   analyses. Hence, the decline appeared to specifically affect the subpopulation of PAH

429   degraders, maybe because phenanthrene became less bioavailable. Although significant

430   amounts of residual phenanthrene were detected in microcosms after 5 and 14 days, the time

431   course of mineralization suggested that it became limiting at day 5, perhaps due to its

432   sequestration or sorption on soil particles. In the SIP200 experiment, the lower dose of

433   phenanthrene likely elicited slower growth, and might have sustained PAH degraders for a

434   longer time. Hence, our results provide evidence that the subpopulation of degraders undergo

435   relatively rapid dynamic changes in response to the level of PAHs available in soil.

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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

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 ($\triangle$;

552 SIP200) or 337 ppm ($\blacktriangle$; 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 ($\blacklozenge$). Other experimental conditions included addition of 34 ppm unlabelled

555 phenanthrene ($\square$), no addition ($\blacksquare$) and abiotic control ($\bigcirc$). 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 ($\blacksquare$), 337 ppm

565 ($\blacktriangle$) or background level ($\bullet$). 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

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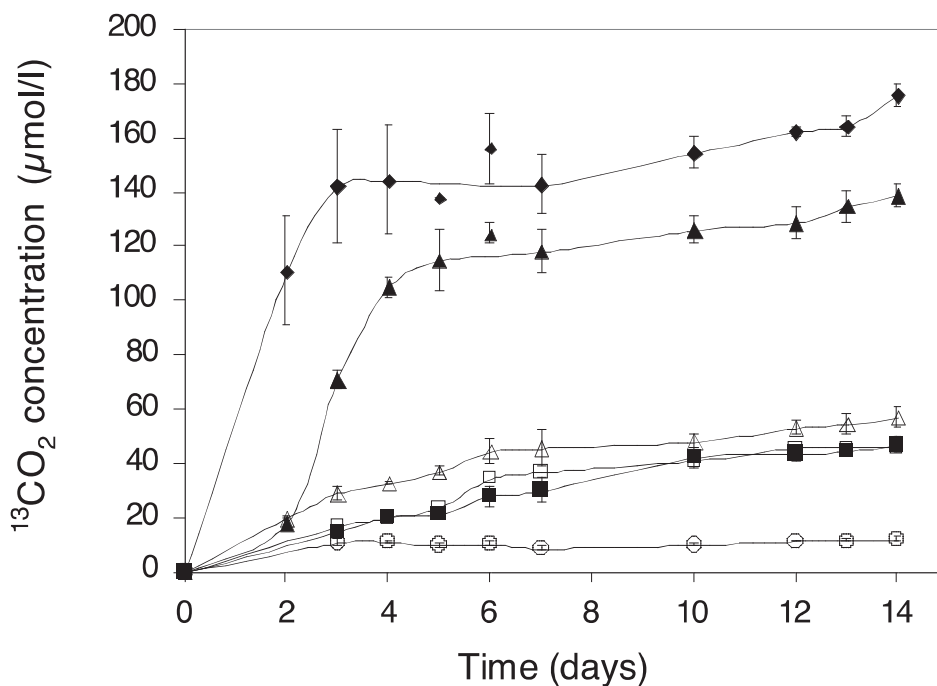
598   gene as an outgroup sequence.

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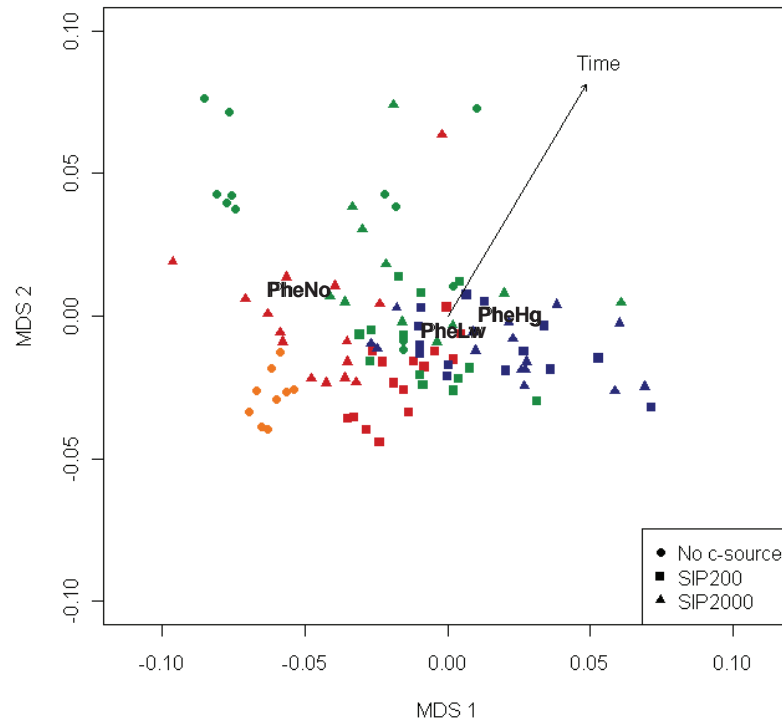
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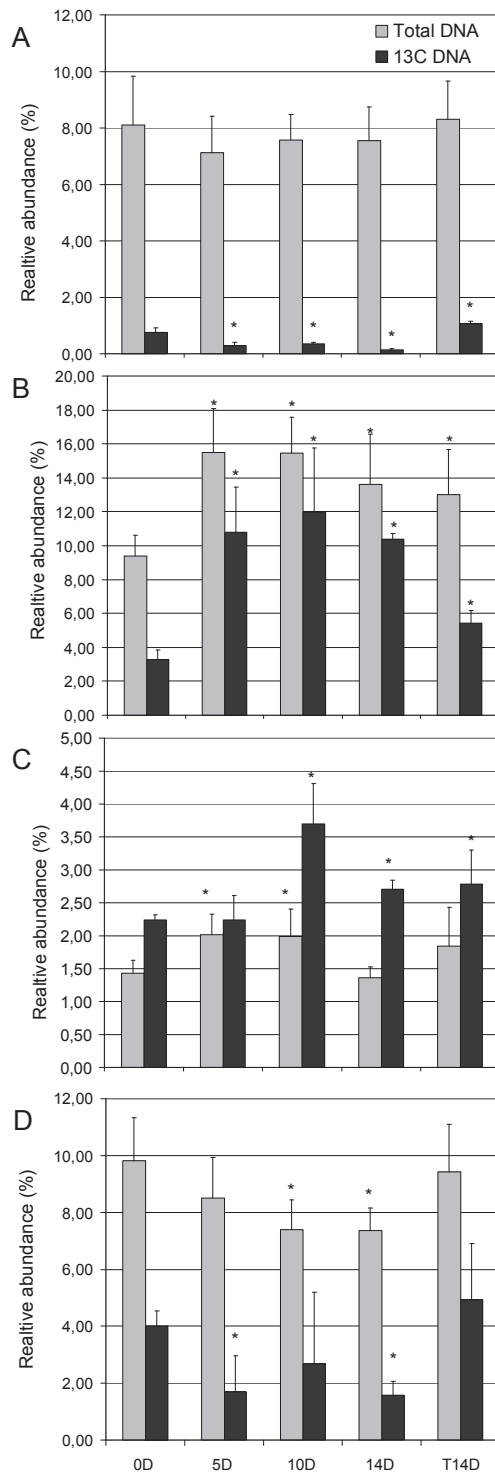
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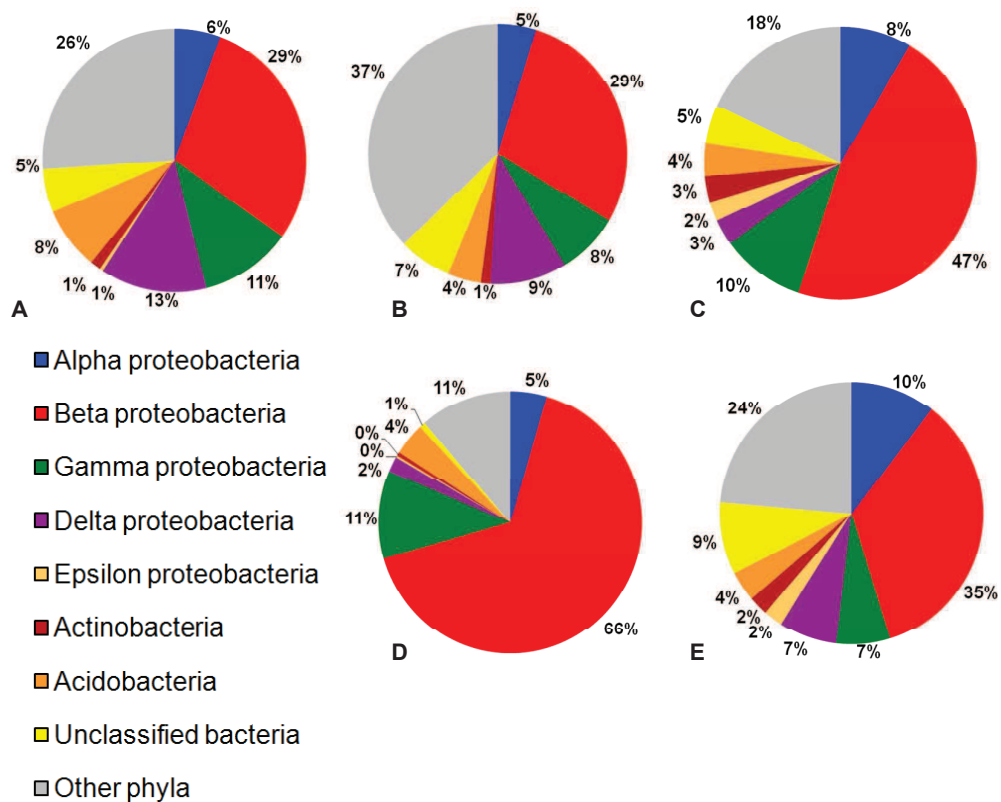
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Chart pies represent sequence repartition into main taxa for the following 16S rRNA gene libraries: A, SIP1 0D (control); B, SIP200 5D 13C; C, SIP200 14D 13C; D, SIP2000 5D 13C; E, SIP2000 14D 13C. "Other phyla" include *Bacteroidetes, Chloroflexi,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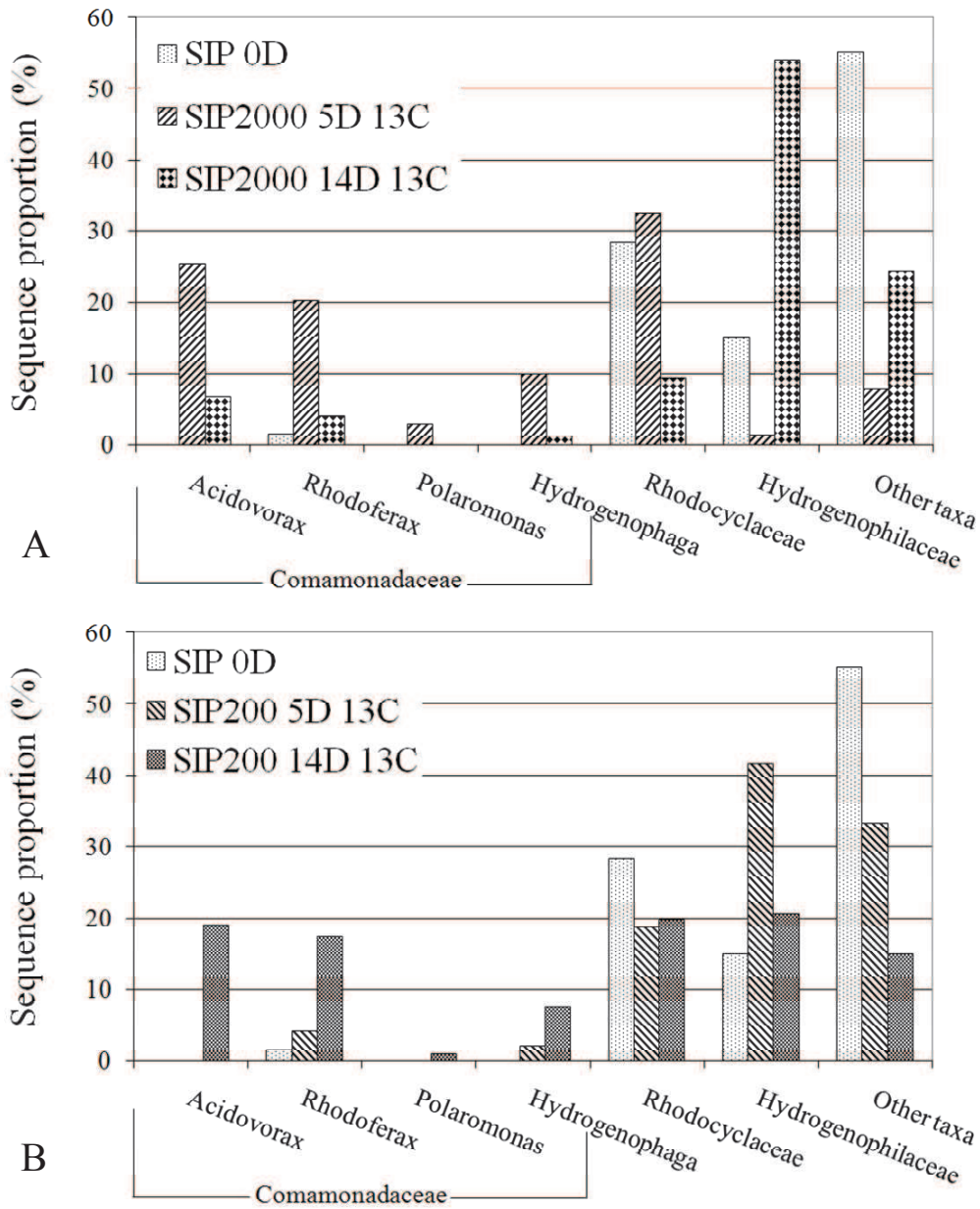
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Sequences were from ^{13}C-DNA libraries of the SIP2000 (A) and the SIP200 (B) experiments,

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
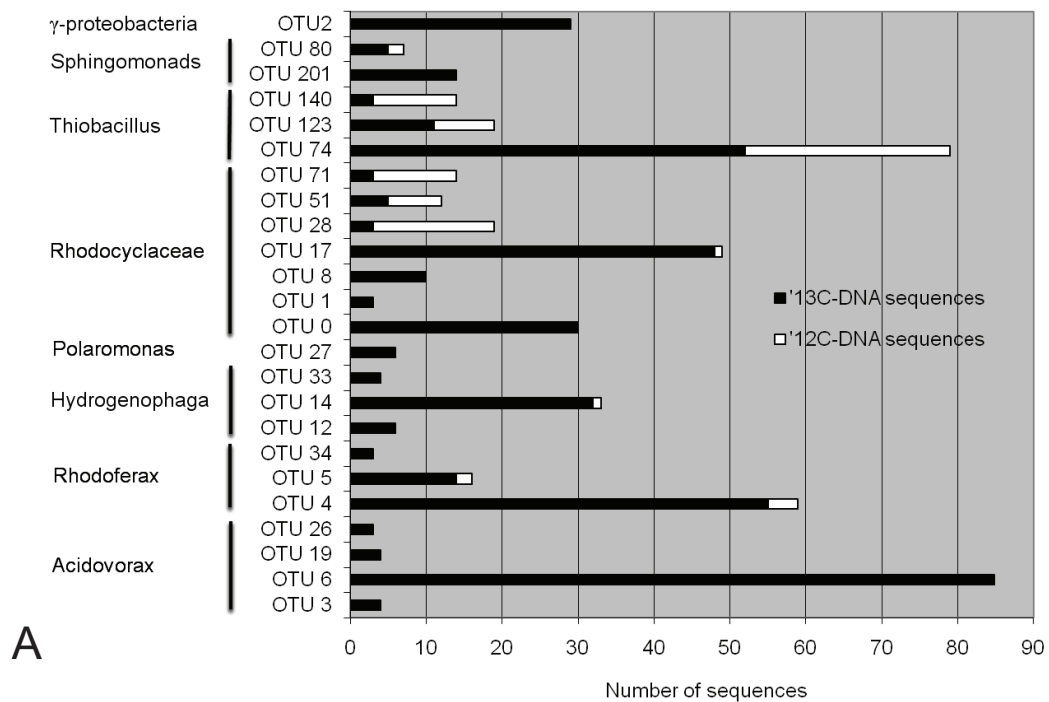
each library.
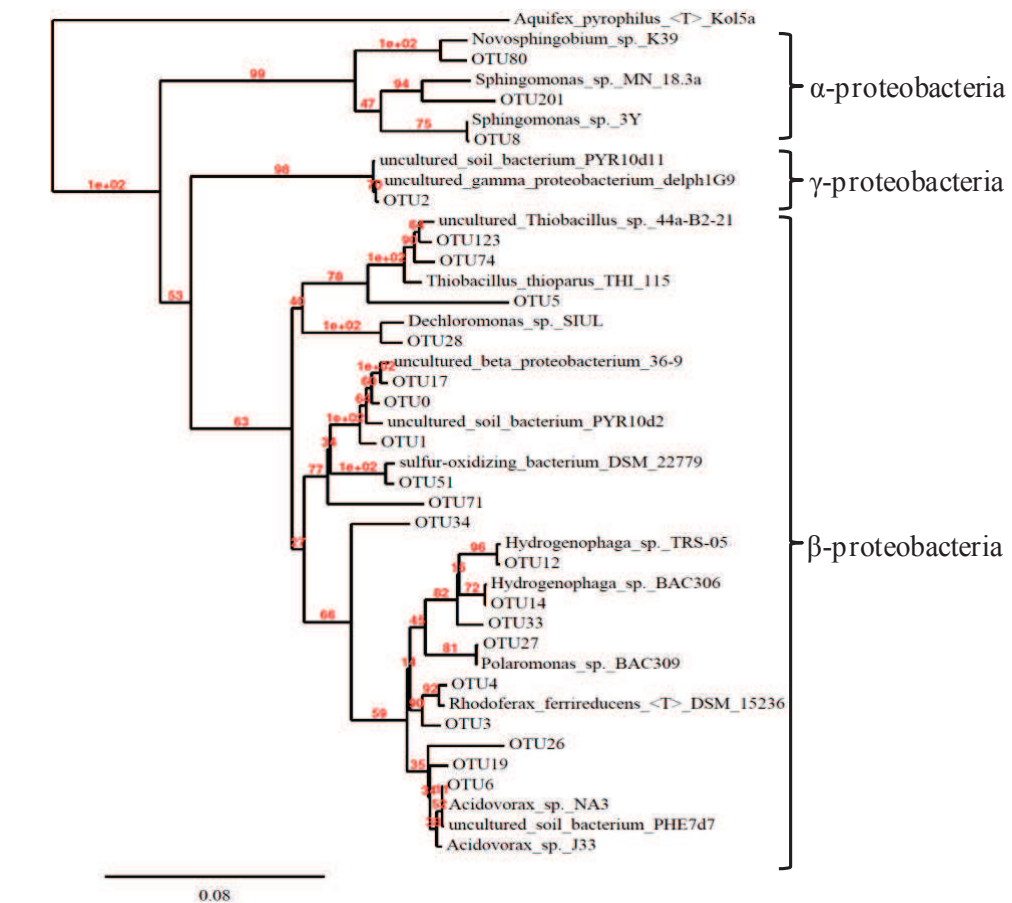
Figure 6

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