Distribution of the *Mycobacterium* community and polycyclic aromatic hydrocarbons (PAHs) among different size fractions of a long-term PAH-contaminated soil

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Summary

Mycobacterium is often isolated from polycyclic aromatic hydrocarbon (PAH)-contaminated soil as degraders of PAHs. In model systems, *Mycobacterium* shows attachment to the PAH substrate source, which is considered to be a particular adaptation to low bioavailability as it results into increased substrate flux to the degraders. To examine whether PAH-degrading *Mycobacterium* in real PAH-contaminated soils, in analogy with model systems, are preferentially associated with PAH-enriched soil particles, the distribution of PAHs, of the PAH-mineralizing capacity and of *Mycobacterium* over different fractions of a soil

with an aged PAH contamination was investigated. The clay fraction contained the majority of the PAHs and showed immediate pyrene- and phenanthrene-mineralizing activity upon addition of ¹⁴C-labelled pyrene or phenanthrene. In contrast, the sand and silt fractions showed a lag time of 15–26 h for phenanthrene and 3–6 days for pyrene mineralization. The maximum pyrene and phenanthrene mineralization rates of the clay fraction expressed per gram fraction were three to six times higher than those of the sand and silt fractions. Most-probable-number (MPN)-polymerase chain reaction demonstrated that *Mycobacterium* represented about 10% of the eubacteria in the clay fraction, while this was only about 0.1% in the sand and silt fractions, indicating accumulation of *Mycobacterium* in the PAH-enriched clay fraction. The *Mycobacterium* community composition in the clay fraction represented all dominant *Mycobacterium* populations of the bulk soil and included especially species related to *Mycobacterium pyrenivorans*, which was also recovered as one of the dominant species in the eubacterial communities of the bulk soil and the clay fraction. Moreover, *Mycobacterium* could be identified among the major culturable PAH-degrading populations in both the bulk soil and the clay fraction. The results demonstrate that PAH-degrading mycobacteria are mainly associated with the PAH-enriched clay fraction of the examined PAH-contaminated soil and hence, that also in the environmental setting of a PAH-contaminated soil, *Mycobacterium* might experience advantages connected to substrate source attachment.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) form a class of toxic hydrophobic organic contaminants with a low bioavailability and a slow biodegradation in soil due to their low water solubility and high sorption to soil particles. Due to PAH ageing, long-term PAH-contaminated soils mostly contain only PAHs with low bioavailability. The PAHs are either strongly sorbed on the organic fraction of the soil or buried in pores inaccessible to microbes. Therefore,

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from a microbial perspective, PAH-contaminated soils and in particular soils containing an aged PAH contamination can be considered oligotrophic (Wick *et al.*, 2003a) and harsh environments for microbes to proliferate.

Bacterial strains isolated from PAH-contaminated soils for their ability to use PAHs as the sole source of carbon and energy belong to a limited number of taxonomic groups such as *Sphingomonas*, *Burkholderia*, *Pseudomonas*, *Rhodococcus* and *Mycobacterium*. *Mycobacterium* especially seems to be an important organism. It has often been reported to degrade high-molecular-weight (HMW) PAHs, i.e. PAHs with more than three rings such as pyrene and benzo[a]pyrene, with an extremely low water solubility (Kanaly and Harayama, 2000; Wick *et al.*, 2001a). Moreover, recent reports on the physiology of PAH-degrading *Mycobacterium* and on the ecology of PAH-contaminated soils suggest that *Mycobacterium* is a specialist in proliferating in the oligotrophic environment of PAH-contaminated soil. *Mycobacterium* seems to be specialized in degrading sorbed PAHs as it has been preferentially selected from soils over other bacteria in enrichment systems with reduced bioavailable phenanthrene sorbed to model hydrophobic sorbents (Bastiaens *et al.*, 2000; Friedrich *et al.*, 2000; Grosser *et al.*, 2000). In addition, *Mycobacterium* shows higher rates of mineralization of phenanthrene sorbed to hydrophobic polymers than expected from abiotic desorption rates (M. Uytendaele and D. Springael, unpublished; Wells *et al.*, 2005). Different reports indicate different physiological adaptations of *Mycobacterium* to oligotrophy and low PAH bioavailability. *Mycobacterium frederiksbergense* LB501T displays multiple substrate utilization (Wick *et al.*, 2003a), low maintenance requirements (Wick *et al.*, 2002) and a high specific affinity for anthracene (Wick *et al.*, 2001b; 2002). Moreover, the LB501T strain attaches to and forms biofilms on anthracene crystals (Wick *et al.*, 2002) and was found to attach to anthracene containing model sorbents (Wells *et al.*, 2005). Attachment to the substrate source is thought to result in an efficient pollutant depletion at low concentrations at the cell surface, steeper concentration gradients and higher substrate transfer rates (Wick *et al.*, 2001a), as such, optimizing substrate bioavailability. Also other *Mycobacterium* strains and PAH degraders from other bacterial genera have been reported to attach on the PAH substrate source (Stelmack *et al.*, 1999; Bastiaens *et al.*, 2000; Wattiau *et al.*, 2002). Such features might play a role in the fitness and ecology of *Mycobacterium* in PAH-contaminated soils. Recently, it was found that *Mycobacterium* was present in relatively higher numbers in PAH-contaminated soils containing low concentrations of mainly HMW PAHs and low-bioavailable PAHs than in soils containing higher concentrations of mainly low-molecular-weight PAHs, i.e. PAHs with less than four rings such as phenanthrene (Leys *et al.*, 2005).

Linking the physiological characteristics of PAH-degrading bacteria regarding PAH degradation, such as substrate source attachment, studied in the laboratory to their ecology in historically PAH-contaminated soils is of importance for interpreting the role of these features in the environmental habitat of these bacteria and the role of these bacteria in PAH bioattenuation in PAH-contaminated soils. In this context, we studied the question whether or not in a PAH-contaminated soil, *Mycobacterium* is also associated with the PAH substrate source as observed in laboratory model systems. It was postulated that as in the lab model systems, attachment to the PAH substrate source might be an ecological advantage for PAH-degrading bacteria and therefore, contributes to their competence in the oligotrophic environment of a PAH-contaminated soil. It would not only increase substrate flux as reported above but would also allow attached bacteria to be 'first' in line to take up the rare PAH substrate molecules desorbing from the soil.

To study this, a long-term PAH-contaminated soil was fractionated by low-energy sonication into four particle-size fractions and the distribution of the *Mycobacterium* community in the different fractions was examined and related to the PAH distribution and the PAH-mineralizing capacity over the different soil fractions. The fractionation procedure was previously validated and applied to investigate enzyme activity in particle-size fractions (Stemmer *et al.*, 1998) and for 16S rRNA-based community analysis in soil fractions (Kandeler *et al.*, 2000; Sessitsch *et al.*, 2001). The PAH-mineralizing capacity of the indigenous microbial community in the soil fractions was examined by mineralization experiments with ¹⁴C-phenanthrene and ¹⁴C-pyrene, while the proportion of *Mycobacterium* in the eubacterial community in the different soil fractions was estimated by most-probable-number (MPN)-polymerase chain reaction (PCR). Phenanthrene and pyrene degraders were estimated in the soil fractions by the WST-1 MPN method (Johnsen *et al.*, 2002), based on growth on PAHs and *Mycobacterium* was identified in the enriched PAH-degrading communities by specific fluorescent *in situ* hybridization (FISH).

Results and discussion

Physicochemical characteristics of soil fractions

Based on dispersion with low-energy sonication (200 J per gram dry soil), the soil contained 11% clay (Table 1). The clay fraction had the highest content of total carbon (97 g kg⁻¹) and total nitrogen (6 g kg⁻¹) of all soil fractions. The C/N ratio decreased from sand to silt to clay. An increasing C and N content and a decreasing C/N ratio from coarse- to fine-sized soil fractions were also observed in several other studies (Jocteur Monrozier

Table 1. Physicochemical characteristics of the examined bulk soil and the particle-size fractions.

Fraction ^a	Particle-size distribution (%)	Total C (g kg ⁻¹)	Total N (g kg ⁻¹)	C/N ^b	PAH concentration (mg kg ⁻¹)		
					LMW ^c	HMW ^d	Total
Bulk soil	–	31.2	1.3	25	4	88	92
Coarse sand	37.4	23.8	0.4	63	1	18	19
Fine sand	29.8	12.2	0.2	55	1	20	21
Silt	18.7	35.5	1.2	30	4	84	88
Clay	10.7	96.6	6.4	15	11	405	416

a. Particle sizes: bulk soil (< 2 mm); coarse sand (2000–200 µm); fine sand (200–53 µm); silt (53–2 µm); clay (2–0.1 µm).

b. Ratio of total C (g kg⁻¹) to total N (g kg⁻¹).

c. LMW: low-molecular-weight PAHs: less than four rings of 16 EPA-PAHs.

d. HMW: high-molecular-weight PAHs: more than three rings of 16 EPA-PAHs.

et al., 1991; Stemmer *et al.*, 1998; 1999; Kandeler *et al.*, 1999; Amellal *et al.*, 2001a). The PAHs present in the bulk soil and in the fractions consisted almost exclusively (96%) of HMW PAHs. Eighty-one per cent of the total PAH content in the bulk soil was recovered in the fractions. Clay contained the largest fraction (49%) of the PAHs present in the bulk soil, compared with coarse sand, fine sand and silt that contained 8%, 7% and 18% of the PAHs respectively. This is in analogy with other reports where the fine silt fraction (20–2 µm) and the clay fraction (< 2 µm) of PAH-contaminated soils were found to contain a higher PAH concentration than the sand fraction (Amellal *et al.*, 2001a,b; Krauss and Wilcke, 2002). The enrichment of the PAHs in the finer fractions of the soil can be explained by multiple reasons such as protection to biodegradation in microaggregates (Zweerts, 2005), the availability of a high specific surface for sorption, the high local nanoporosity for sequestration or the high content of hydrophobic organic matter (Xing and Pignatello, 1996; Chung and Alexander, 1998; 2002; Bogan and Sullivan, 2003). In our study, the high total C content (97 g kg⁻¹), which is related to the organic C content, in the clay fraction can most probably explain the accumulation of PAHs in this fraction as several studies have acknowledged the important role of organic C in PAH sequestration. On the other hand, PAHs are not always connected with the clay fraction as also the texture of the PAH-containing waste (ashes,

slags, rubble) that was initially deposited in the soil is important for the PAH distribution among particle-size fractions (Krauss and Wilcke, 2002). In our study, the clay fraction clearly contained most of the PAHs. As a result, the clay fraction can be seen as the major environmental PAH substrate stock for potential PAH degraders in the examined PAH-contaminated soil.

Mineralization of ¹⁴C-phenanthrene and ¹⁴C-pyrene in soil fractions

The mineralization of ¹⁴C-phenanthrene and ¹⁴C-pyrene to ¹⁴CO₂ in the bulk soil and the particle-size fractions was measured to examine the PAH-degrading capacity of the indigenous microbial community in the different soil fractions (Fig. 1). Obtained kinetic parameters are shown in Table 2. The relatively high final extent of mineralization (46–67%) suggests the complete consumption of the ¹⁴C-PAHs, being the rest probably converted into biomass and biodegradation products different from CO₂ (Garcia-Junco *et al.*, 2003). The community in the clay fraction immediately initiated pyrene mineralization, while a lag phase of 3.5–5.9 days was observed before the communities in the silt and sand fractions started pyrene mineralization (Table 2). The mineralization of ¹⁴C-phenanthrene also started immediately in the clay fraction, while silt and sand showed a lag phase of 15–26 h. No significant difference

Table 2. Maximum mineralization rate and lag time for phenanthrene and pyrene mineralization by the indigenous microbial community in the bulk soil and the particle-size fractions.

Fraction	Maximum mineralization rate (% h ⁻¹)		Lag time (h)	
	Phenanthrene	Pyrene	Phenanthrene	Pyrene
Bulk soil	1.40 B	1.05 AB	4.5 C	9.9 D
Coarse sand	0.80 B	0.35 C	25.5 A	114.0 B
Fine sand	0.95 B	0.45 BC	22.7 AB	141.9 A
Silt	0.81 B	0.35 C	14.7 B	83.9 C
Clay	4.45 A	1.54 A	0.4 C	1.9 D

The results are the mean of three replicates. Means within one column with the same letter are not significantly different by a Tukey test ($P < 0.05$).

Fig. 1. Mineralization of ^{14}C -phenanthrene and ^{14}C -pyrene (as $^{14}\text{CO}_2$) by the indigenous microbial community in the bulk soil and the particle-size fractions. Error bars represent standard errors from three replicates.

in lag time of phenanthrene and pyrene mineralization was observed between clay and the bulk soil. The maximum mineralization rate of phenanthrene and pyrene was significantly different for the clay and sand-silt communities (Table 2). First of all, these results show that despite the aged nature of the PAH contamination in the used soil, the soil contained and sustained a highly active PAH-degrading community. Similar results have been obtained with other PAH-contaminated soils (Johnsen and Karlson, 2006). Although freshly added PAHs do not behave in the same way in soil as aged PAHs (Hatzinger and Alexander, 1995; Carmichael *et al.*, 1997; Alexander, 2000) and therefore, the relative fast mineralization of the freshly added ^{14}C -labelled PAHs cannot be extrapolated to degradation of native PAHs in the soil, it might indicate that in nature desorbing degradable PAH compounds are rapidly degraded by the residing community. Second, we show that the PAH-mineralizing activity in the soil was especially associated with the clay fraction. It shows that

the used fractionation procedure retained the PAH-mineralizing activity of the bulk soil in the soil fractions and that different PAH-mineralizing activities calculated per gram fraction exist between them. The mineralization curves for the sand and silt fractions were sigmoidal in nature, which indicated that a period of growth of PAH-degrading organisms was needed before significant mineralization of the added ^{14}C -PAHs occurred (Grosser *et al.*, 1995; MacLeod and Semple, 2002). In contrast, direct mineralization was observed for the clay fraction, indicating that the clay fraction contained higher numbers of PAH mineralizing populations than the other fractions. This accounted especially for pyrene mineralizing populations. Our data show that the PAH-degrading capacity is localized in the clay fraction of the PAH-contaminated soil and is therefore connected with the PAH substrate source. As PAH-degrading bacterial isolates able to use pyrene as sole source of carbon and energy are almost exclusively *Mycobacterium*, the high pyrene-degrading capacity observed in the clay fraction, is a first indication of the presence of high numbers of PAH-mineralizing *Mycobacterium* in the PAH-containing clay fraction.

MPN-PCR estimate of *Mycobacterium* in soil fractions

A *Mycobacterium*-specific nested PCR (Leys *et al.*, 2005) was applied as a MPN-PCR to estimate the number of *Mycobacterium* cells within the eubacterial community in the different soil fractions (Fig. 2). The use of a MPN-PCR approach to enumerate bacterial populations in soil is prone to errors and is therefore not an absolute quantifi-

Fig. 2. Most-probable-number (MPN)-PCR estimate of the 16S rRNA gene copy number of eubacteria and *Mycobacterium* for the bulk soil and the particle-size fractions. Means of log MPN (two replicates) with a different uppercase or lowercase letter are significantly different by a Tukey test ($P < 0.05$) for eubacteria or *Mycobacterium* respectively.

Fig. 3. UPGMA dendrogram of 16S rRNA-based PCR-DGGE community profiles of eubacteria in the different soil fractions of the examined PAH-contaminated soil. Replicates of soil fractionation are indicated by A and B. Soil DNA, used as template for PCR amplification, was diluted 10-fold. The scale indicates the similarity level (0–100%).

cation (Fredslund *et al.*, 2001). However, the numbers provided for *Mycobacterium* and eubacteria are indicative for the relative proportions of both bacterial groups in the different soil fractions. The eubacterial log MPN count (as an estimate of the eubacterial 16S rRNA gene copies per gram soil) for the clay fraction was 6.5. No significant difference in eubacterial MPN count between clay and the other fractions was found. The log MPN count for *Mycobacterium* (as an estimate of the *Mycobacterium* 16S rRNA gene copies per gram soil) in the clay fraction was 5.3. This log MPN was significantly different from sand and silt fractions, but not from the bulk soil. The number of 16S rRNA gene copies in prokaryotic genomes can vary from one to 15 copies (Klappenbach *et al.*, 2001), while *Mycobacterium* has one to two copies (Bercovier *et al.*, 1986; Leys *et al.*, 2005). The eubacterial communities in the bulk soil and the different soil fractions seem to be dominated by α -proteobacteria and actinomycetes (see below) which both have an average rRNA gene copy number of two (Klappenbach *et al.*, 2001). Hence, assuming an average 16S rRNA gene copy number for the eubacterial and *Mycobacterium* community of two, mycobacteria represented about 10% of the eubacteria in the

clay fraction, while this was only about 0.1% in the sand and silt fractions. These results showed that the *Mycobacterium* community present in the bulk soil was proportionally more concentrated in the clay fraction of the examined PAH-contaminated soil than in the other fractions.

Community analysis of indigenous eubacteria and *Mycobacterium* in soil fractions

The unweighted pair group method using arithmetic averages (UPGMA) dendrogram of the eubacterial community of the bulk soil and the particle-size fractions is shown in Fig. 3. The eubacterial community structure within the soil clearly depended on the particle-size fraction. The eubacterial community of the clay fraction was different from sand and silt fractions and represented the dominant soil community with a similarity [$\pm$ standard deviation (SD)] between the bulk soil and clay of $80 \pm 1\%$. The dominant populations in the bulk soil and clay fraction were identified by sequencing relevant 16S rRNA gene bands in the denaturant gradient gel electrophoresis (DGGE) profile (Fig. 3) and analysis of the sequences. The outcome of the analysis is shown in Table 3 and shows that the dom-

Table 3. Closest GenBank match and sequence similarity of selected 16S rRNA gene-based DGGE bands.

Band ^a	Accession No.	Closest GenBank match (accession No.)	Similarity (%)
K3	AM085778	<i>Bradyrhizobium</i> sp. BTA-1 (AJ558025)	98.4
K4	AM085779	<i>Porphyrobacter tepidarius</i> DSM10594 (AF465839)	97.9
K5	AM085780	Uncultured α -proteobacterium clone 406T3 (DQ110117)	95.6
K6	AM085781	<i>Arthrobacter</i> sp. M4 (AY177360)	99.5
K7	AM085782	<i>Mycobacterium pyrenivorans</i> DSM44605 (AJ431371)	99.0
K10	AM085785	<i>Mycobacterium pyrenivorans</i> DSM44605 (AJ431371)	98.8
K11	AM085786	<i>Mycobacterium tusciae</i> JS617 (AF498655)	98.8
K12	AM085787	<i>Mycobacterium tusciae</i> FI-25796 (AF058299)	98.8
K13	AM085788	<i>Mycobacterium tusciae</i> JS617 (AF498655)	98.8

a. Bands K3–K7: PCR of the 16S rRNA gene with eubacterial primers GC40-63f and 518r; bands K10–K13: PCR of the 16S rRNA gene with *Mycobacterium*-specific primers MYCO66f and GC40-MYCO600r.

Fig. 4. UPGMA dendrogram of 16S rRNA-based PCR-DGGE community profiles of *Mycobacterium* spp. in the examined PAH-contaminated bulk soil and its clay fraction. Replicates of soil fractionation are indicated by A and B. Soil DNA, used as template for PCR amplification, was diluted 10-fold (indicated by 1) or 100-fold (indicated by 2). The scale indicates the similarity level (0–100%).

inant bacterial species in the soil and the clay fraction were related to species such as an uncultured α -proteobacterium (band K5), *Bradyrhizobium* (band K3), *Porphyrobacter* (band K4), *Arthrobacter* (band K6) and *Mycobacterium pyrenivorans* (band K7). It shows that next to *Mycobacterium*, also other bacteria are concentrated in the clay fraction. This was not unexpected; several studies have indicated that microbial biomass concentrates in the silt and clay fractions of a soil (Jocteur Monrozier *et al.*, 1991; Van Gestel *et al.*, 1996; Stemmer *et al.*, 1998; Kandeler *et al.*, 2000; Sessitsch *et al.*, 2001) as organic matter and clay particles provide substrates and nutrients for the survival of bacteria (Van Gestel *et al.*, 1996). Interestingly, *M. pyrenivorans* is a recently described PAH-degrading *Mycobacterium* species that can use pyrene as sole source of carbon and energy (Derz *et al.*, 2004). Moreover, *M. pyrenivorans* was also identified as the dominant *Mycobacterium* population within the *Mycobacterium* community (band K10) (Table 3). The dendrogram of the *Mycobacterium* community showed a high similarity ($\pm$ SD) of $92 \pm 3\%$ between the bulk soil and clay (Fig. 4). The DGGE fingerprints of the *Mycobacterium* community in the sand and silt fractions showed a weak signal and were therefore excluded from the dendrogram. All other bands within the *Mycobacterium* community (K11–K13) corresponded to 16S rRNA gene sequences of *Mycobacterium tusciae*. Interestingly, mycobacteria related to *M. tusciae* have also been found in different other long-term PAH-contaminated soils using culture-independent techniques (Leys *et al.*, 2005). In contrast, up to now, mycobacteria related to *M. tusciae* have never been cultured as PAH degrader from soils. This can possibly be explained by the slow growth (4 weeks) of *M. tusciae* in currently used culture media (Tortoli *et al.*, 1999).

Most-probable-number estimate of culturable PAH degraders in soil and *Mycobacterium* identification by FISH

To link community structure and functionality of *Mycobacterium* in soil K3663, the number of phenanthrene and pyrene degraders in the soil fractions were estimated by the WST-1 MPN method and the presence of *Mycobacte-*

rium among the culturable PAH-degrading bacteria was examined by FISH. The log MPN count of the PAH degraders (per gram soil) in the clay fraction was 7.5 for phenanthrene and 6.6 for pyrene. These log MPN counts were significantly higher than those of the sand and silt fractions, but were similar to those of the bulk soil (Fig. 5). The amount of pyrene degraders in fine sand was lower than the detection limit of 10^2 cells per gram soil. Therefore, log MPN of pyrene degraders for fine sand were excluded from the Tukey test. The log MPN count for phenanthrene degraders in the clay fraction was 1 log unit higher than the amount of eubacteria in the clay fraction, as estimated with MPN-PCR. As mentioned above, MPN-PCR does not give absolute numbers and can underestimate the actual size considerably due to factors such as DNA extraction efficiency and PCR efficiency (Fredslund *et al.*, 2001). On the other hand, also the WST-1 MPN method probably underestimates the numbers of PAH degraders by detecting only those cells able to grow in liquid culture with phenanthrene or pyrene as sole source of carbon and energy. In addition, the MPN method does

Fig. 5. Most-probable-number (MPN) estimate of phenanthrene and pyrene degraders for the bulk soil and the particle-size fractions. Means of log MPN (two replicates) with a different uppercase or lowercase letter are significantly different by a Tukey test ($P < 0.05$) for phenanthrene or pyrene degraders respectively.

not take into account co-metabolism of PAHs. This underestimation was demonstrated by the existence of a ^{14}C -pyrene mineralization capacity in the fine sand fraction, while no pyrene utilizers were found in this fraction by the MPN count, as is in accordance with similar results of Johnsen and Karlson (2006).

The PAH degraders in the MPN microplates were examined for the presence of *Mycobacterium* by peptide nucleic acid (PNA)-FISH, using specific probes for *Mycobacterium* species related to either *Mycobacterium austroafricanum*, *M. frederiksbergense* or *M. gilvum* (Table 4). These three *Mycobacterium* spp. are known to include most of the described PAH-degrading *Mycobacterium* strains (Leys et al., 2005). rRNA gene sequence analysis assigned *M. pyrenivorans* to the 16S rRNA gene subgroup that contains also *M. austroafricanum* (Derz et al., 2004). Moreover, the sequence of the PNA probe specific for *M. austroafricanum* matched completely with the 16S rRNA sequence of the type strain of *M. pyrenivorans* and hence the PNA probe will also target *M. pyrenivorans*. *Mycobacterium tusciae* is not a target of these FISH probes. *Mycobacterium austroafricanum*/*M. pyrenivorans*-related species proved to be important phenanthrene-degrading populations in silt (48% of all phenanthrene-degrading cells) and clay (17% of all phenanthrene-degrading cells). This indicates that *M. pyrenivorans* recovered as most dominant *Mycobacterium* population on the basis of 16S rRNA gene analysis (see above) is also a dominant phenanthrene-degrading population in the soil and the clay fraction. *Mycobacterium frederiksbergense* was recognized as an important pyrene-degrading population in the clay fraction (24% of all pyrene-degrading cells). No cells in the microplates were identified as *M. gilvum*. The observation that *M. frederiksbergense* was present among the culturable PAH degraders is in contrast with the data from the 16S rRNA gene analysis of the *Mycobacterium* community in which *M. frederiksbergense* was not present. We were not able to recover all *Mycobacterium* bands from the *Mycobacterium* DGGE fingerprints as clones suitable for sequencing and it might be that the bands missing among the clones represented *M. frederiksbergense*. Alterna-

tively, as found for other PAH-contaminated soils, using a PCR approach specifically targeting *M. frederiksbergense*, *M. frederiksbergense* might be present at concentrations in the soil which were too low for becoming a visible dominant population on the *Mycobacterium* 16S rRNA gene DGGE profile (N.M. Leys and D. Springael, unpublished). Reports indicate that populations proportionally present at 1% and less might be missed by the PCR-DGGE approach (Muyzer et al., 1993). *Mycobacterium*-specific FISH identified only 19–43% of the culturable PAH-utilizing cells in the bulk soil and the clay fraction as *Mycobacterium* spp. The other PAH-degrading cells might belong to other species of *Mycobacterium*. Unfortunately, a FISH probe directed towards other *Mycobacterium* species or towards the complete *Mycobacterium* genus is not available. Alternatively, members of other bacterial genera might have been present among the PAH degraders in the wells. Indeed, next to *Mycobacterium*, other bacterial genera have been described to degrade and grow on phenanthrene. In contrast, bacteria able to utilize pyrene are almost exclusively *Mycobacterium*. Finally, bacteria not able to attack PAHs but metabolically associated with PAH-degrading bacteria in food webs might have developed in the wells. Also the highly similar eubacterial community DGGE fingerprints of the clay fraction and the bulk soil show that other types of bacteria different from *Mycobacterium* occupy the PAH-enriched clay fraction. The pyrene degraders in the coarse sand and silt fractions could not be identified by FISH, as sufficient cells for identification by FISH could not be recovered from the pyrene-coated wells. This is in analogy with the PAH-degrading activity measurements where pyrene degradation in the sand and silt fractions was only observed after lag phases of 3–6 days. These results show that the *Mycobacterium* populations that accumulated in the PAH-enriched clay fraction consist at least partially of PAH degraders.

Conclusions

Our results show that in the examined long-term PAH-contaminated soil, PAH-degrading *Mycobacterium*,

Table 4. Identification of phenanthrene and pyrene degraders by *Mycobacterium*-specific FISH.

FISH probes ^a	Phenanthrene (%) ^b					Pyrene (%) ^b	
	Bulk soil	Coarse sand	Fine sand	Silt	Clay	Bulk soil	Clay
<i>Mycobacterium austroafricanum</i>	42	3	0	48	17	6	9
<i>Mycobacterium frederiksbergense</i>	1	0	0	6	2	25	24

a. Probe for *Mycobacterium gilvum* gave no positive results.

b. The identification of PAH degraders is expressed as a relative percentage of *Mycobacterium* cells (visualized by specific FISH) to eubacterial cells (visualized by DAPI). No sufficient sample could be prepared for pyrene degraders in coarse sand and silt; no pyrene degraders in fine sand. The pyrene numbers for bulk soil and clay are for one of the two replicates; no sufficient sample could be prepared for the other replicate.

among other bacteria, was clearly associated with the clay fraction that formed the main storage fraction of PAHs in the soil. *Mycobacterium* represented about 10% of the eubacteria in the clay fraction, while this was only about 0.1% in the sand and silt fractions, showing that the *Mycobacterium* community present in the bulk soil was proportionally more concentrated in the clay fraction of the examined PAH-contaminated soil than in the other fractions. Moreover, *Mycobacterium* was among the main pyrene- and phenanthrene-utilizing populations in the soil, and a high pyrene mineralization activity, an activity almost exclusively associated with PAH-degrading *Mycobacterium* isolates, was registered in the clay fraction. The association of *Mycobacterium* with the PAH-containing soil fraction suggests attachment of *Mycobacterium* to PAH-containing soil particles and seems to be in analogy with attachment and biofilm formation of *Mycobacterium* on PAH crystals and model sorbents (Bastiaens *et al.*, 2000; Wick *et al.*, 2002; Wells *et al.*, 2005). The association of bacteria with organic pollutant contaminated clay particles has been shown before. Lünsdorf and colleagues (2000) reported on the appearance of aggregates consisting of bacteria surrounded by polychlorinated biphenyl (PCB)-containing clay leaflets in an artificial set-up. As proposed for attachment/biofilm formation of *Mycobacterium* on PAH crystals and PAH-containing model sorbents, PAH-degrading *Mycobacterium* might be benefiting from this situation by increasing PAH substrate flux and hence PAH bioavailability (Wick *et al.*, 2001a). In addition, *Mycobacterium* cells may also have direct access to slow-desorbing PAH compounds (Wells *et al.*, 2005). Moreover, this fraction provides other food sources (from organic carbon) and nutrients (Kandeler *et al.*, 2000; Sessitsch *et al.*, 2001) which might be of importance to remain active and competitive in the oligotrophic environment (Wick *et al.*, 2003a). However, the question remains whether the association of *Mycobacterium* with PAH-containing substrata in soil is simply due to the fact: (i) that clay is a preferential habitat for *Mycobacterium* or (ii) that the specific enrichment of *Mycobacterium* in the clay fraction of the examined soil is the consequence of a food strategy specifically directed to PAHs. Regarding the first explanation, *Mycobacterium* displays a hydrophobic cell wall that might result into association with organic loaded clay particles (Wick *et al.*, 2003a) or *Mycobacterium* cells may be better protected from protozoa in the clay fraction (Sessitsch *et al.*, 2001). Similarly, *Mycobacterium* has been shown to have low oxygen requirements (Fritzsche, 1994) and clay particles might be a better niche for such organisms. Indeed, Sessitsch and colleagues (2001) showed that clay particles provided a niche for aerobes as well as for anaerobes, whereas larger particle sizes were dominated by aerobic bacteria. Not much research has been performed on the distribution of *Mycobacterium*

in different soil fractions in non-contaminated soils. Sessitsch and colleagues (2001) showed that actinomycetes belonged to the main populations of the clay fraction of an agricultural soil. However, no data on other soil fractions or on relative proportions of actinomycetes to all bacteria were provided. Regarding the second explanation, we recently showed that sliding over a surface by a PAH-degrading *Mycobacterium* strain was strongly increased by distant phenanthrene crystals suggesting PAH-directed motility of *Mycobacterium*, even in the presence of low concentrations of the PAH (L. Nielsen and D. Springael, unpublished). As such, upon contamination of the soil and concentration of the PAHs in the clay fraction, *Mycobacterium* might have been attracted by the PAH food source and build up a population in the clay fraction. Other characteristics of *Mycobacterium* such as high hydrophobicity, low oxygen requirements and multiple substrate utilization might then have been additional advantageous characteristics to compete well with other bacteria at that location for nutrients and even PAHs. Moreover, it was found that *Mycobacterium* adapts its surface depending on the growth substrate. Growth on PAHs supported adhesion to hydrophobic surfaces (including PAHs) yet not glass surfaces (Wick *et al.*, 2002; 2003b).

To differentiate between both explanations, more research is needed applying a similar microscale analysis to other PAH-contaminated and non-contaminated soils. In particular, non-contaminated soils and soils with the PAH contamination concentrated on larger particle-size fractions would be of interest. Krauss and Wilcke (2002) observed a heterogeneous distribution of PAHs among the particle-size fractions of urban soils because the soils received different PAH-contaminated wastes with varying texture, but no research was performed on the microbial ecology of the soils. Interestingly, Amellal and colleagues (2001a) showed that indigenous PAH-degrading bacteria were embedded in the aggregates of the fine silt fraction (20–2 µm), containing most of the PAHs. However, these bacteria were not identified.

Whatever the explanation is, the association of *Mycobacterium* with PAH-containing soil particles might be of importance for natural bioattenuation activities in soil as slowly desorbing PAH molecules might be directly degraded by the nearby PAH-degrading biocatalysts and as such leaching of PAHs might be minimized. Indeed, despite the low amounts of PAHs, presented in a low-bioavailable form in the examined soil, the microbial community in the soil still showed high PAH-mineralization activity for both pyrene and phenanthrene indicating that those populations are ready to degrade desorbing PAH molecules. The fact that, despite these apparently non-selective conditions, a high PAH-degrading population survives and remains active with a high number of *Myco-*

bacterium concentrated around the PAH substrate source is on itself an intriguing study object.

Experimental procedures

Soil sample

Soil sample K3663 was taken from a historically PAH-contaminated site of a former coal gasification plant in Denmark. The soil was sieved at 2 mm and stored fresh for 4 months at 4°C in the dark. Selected physicochemical characteristics of the soil are shown in Table 1. Total carbon and total nitrogen content were measured using a combustion analyser (Vario MAX CN, Elementar Analysensysteme GmbH, Hanau, Germany). The PAH content 16 PAHs listed by the US Environmental Protection Agency (EPA) was measured as described by Leys and colleagues (2005). Soil pH of K3663 was 7.6, as determined in CaCl_2 10^{-2} M (soil/liquid ratio of 1:2.5).

Fractionation procedure

Fractionation of the soil was performed according to the method described by Stemmer and colleagues (1998). Low-energy sonication (200 J per gram soil) of the soil sample in combination with wet sieving and repeated centrifugation resulted into four particle-size fractions, i.e. 2000–200 µm (coarse sand); 200–53 µm (fine sand); 53–2 µm (silt) and 2–0.1 µm (clay). The fractions were kept overnight at 4°C before use.

^{14}C -PAH mineralization by indigenous soil bacteria

Mineralization of [$9\text{-}^{14}\text{C}$]-phenanthrene (Sigma, 13.1 mCi mmol $^{-1}$, dissolved in dichloromethane, radiochemical purity > 98%) or [$4,5,9,10\text{-}^{14}\text{C}$]-pyrene (Sigma, 55 mCi mmol $^{-1}$, dissolved in acetone, radiochemical purity > 98%) by the indigenous microbial community in the bulk soil and the different soil fractions was examined in 15 ml Pyrex tubes. After evaporation of dichloromethane or acetone (100–150 µl) in 250 ml Erlenmeyer flasks, the ^{14}C -PAHs were dissolved in minimal mineralization medium (pH 5.7) (Ortega-Calvo *et al.*, 1995) to have a final radioactivity of 0.007 µCi ml $^{-1}$. The PAH concentration in the mineralization medium was below water solubility, 1.18 mg l $^{-1}$ for phenanthrene or 0.135 mg l $^{-1}$ for pyrene at 25°C (Miller *et al.*, 1985). Unlabelled PAH was not added. PAH-containing mineralization medium (5 ml) was added to 200 mg (dry weight) of soil. The tubes were closed with Teflon-lined stoppers equipped with alkali traps (1 ml of 0.5 M NaOH) to measure $^{14}\text{CO}_2$ produced from ^{14}C -PAHs and incubated at 20°C on a rotary shaker at 100 r.p.m. Periodically, the NaOH solution was removed from the trap and replaced with fresh alkali. The removed NaOH solution was mixed with 5 ml of liquid scintillation cocktail (Ready Safe, Beckman Instruments) and the mixture was kept in darkness for at least 8 h for dissipation of chemiluminescence. Radioactivity was measured with a liquid scintillation counter (model LS6500; Beckman Instruments, Fullerton, CA, USA).

Cumulative mineralization curves were established in triplicate for the bulk soil and each soil fraction. The maximum mineralization rate (% h $^{-1}$) was calculated as the slope of the linear regression line between two successive points of the mineralization curve where the amount of $^{14}\text{CO}_2$ showed the largest increase. The lag time was calculated as the intersection of the x-axis with the linear regression line of maximum mineralization (Broos *et al.*, 2005).

DNA extraction

Total DNA was extracted from the bulk soil and the soil fractions by a method modified from Boon and colleagues (2000). For each soil fraction, 0.4 g of soil material, 0.6 g of beads (0.10–0.11 mm diameter) (B. Braun Biotech International, Melsungen, Germany) and 0.8 ml of 0.1 M Na_3PO_4 (pH 8) were added together in each of three 2 ml microtubes (Greiner Bio-One). The mixtures were beaten for 45 s at speed 6.0 using a Hybaid RiboLyser (Thermo Electron). A lysozyme solution (32 µl) (50 mg ml $^{-1}$ Tris-HCl, 10 mM, pH 9) was added, followed by 30 min head-over-end shaking at 20°C. Subsequently, 60 µl of 20% SDS and 0.2 ml of 8 M ammonium acetate (pH 7.2) were added, followed by 10 min head-over-end shaking at 20°C. The supernatant of the three microtubes was collected after centrifugation at 5916 g for 15 min at 15°C in a 10 ml tube (Novolab, Geraardsbergen, Belgium), resulting in a final DNA extract from 1.2 g of soil. After purification with 2.4 ml of chloroform-isoamylalcohol (24:1), the mixture was shaken for 60 min head-over-end at 20°C and centrifuged at 5916 g for 15 min at 15°C. The aqueous phase was transferred to a new tube and 0.8 volume of isopropanol was added for overnight precipitation of the DNA at –20°C. After centrifugation at 12 074 g for 30 min at 15°C, the pellet was dried at room temperature for 1 h and resuspended in 250 µl of TE buffer (10 mM Tris; 1 mM EDTA; pH 8). The crude DNA extract was purified with the Wizard® DNA clean-up kit with vacuum manifold (Promega), as described by Promega. The purified DNA was finally recovered in 50 µl of TE buffer and stored at –20°C.

Polymerase chain reaction amplification

Eubacterial 16S rRNA gene fragments were amplified by PCR using the eubacterial primers GC40-63f and 518r (El Fantroussi *et al.*, 1999), as described by Moreels and colleagues (2004). *Mycobacterium* 16S rRNA gene fragments were amplified by PCR using the direct or nested PCR approach with *Mycobacterium*-specific primers MYCO66f and GC40-MYCO600r, as described by Leys and colleagues (2005). In the nested PCR approach, the eubacterial primers 27f and 1492r were used in the first PCR and 1 µl of the resulting PCR product was used as template in the second PCR with the MYCO66f and GC40-MYCO600r primers. Polymerase chain reaction amplification was performed on an Eppendorf Mastercycler. The PCR mixture contained 1 µl of soil DNA as template, 1.25 U Taq polymerase (Qiagen), 25 pmol of each primer (Qiagen), 10 nmol of each dNTP (Invitrogen) and 1× PCR buffer (Qiagen) in a total volume of 50 µl.

Most-probable-number (MPN)-PCR estimate of eubacterial and *Mycobacterium* populations in soil

The MPN-PCR enumeration was adapted from Fredslund and colleagues (2001). DNA was extracted from the different soil fractions after fractionation in duplicate. A 10-fold dilution series of the soil DNA extract (to 10^{-6}) was prepared in TE buffer and three times 1 μ l of each dilution was used as template in the *Mycobacterium*-nested PCR described above (three-row MPN). The MPN enumeration of eubacteria was based on the PCR products of the first PCR and MPN enumeration of *Mycobacterium* was based on the PCR products of the second PCR. The PCR products were run at 90 V for 1.25 h in 1.5% agarose gels (Electro-4 Gel Tank, Hybaid Limited, Ashford, UK), containing 0.2 mg l⁻¹ ethidiumbromide and photographed on a UV transillumination table with a gel documentation system (Vilber Lourmat, Cedex, France). The numbers of positive and negative tubes that produced amplification products were scored for each sample. The MPN was calculated by using Microsoft Excel, as described by Briones and Reichardt (1999), and is an estimation of the 16S rRNA gene copy number of eubacteria or *Mycobacterium* in the soil fractions (Fredslund *et al.*, 2001).

Denaturant gradient gel electrophoresis analysis

Denaturant gradient gel electrophoresis was performed on an INGENYphorU-2 system (Ingeny International, Goes, the Netherlands). Denaturant gradient gel electrophoresis analysis of the PCR products from amplification with the eubacterial primers GC40-63f and 518r was performed as described by Moreels and colleagues (2004), but for 15 h at 120 V. Denaturant gradient gel electrophoresis analysis of the *Mycobacterium* PCR products was performed as described by Leys and colleagues (2005). Denaturant gradient gel electrophoresis gels were stained for 30 min with 1 $\times$ SYBR Gold (Molecular Probes, Leiden, the Netherlands) and photographed on a UV transillumination table with a GeneLink camera system (SYNGENE, Cambridge, UK). GelCompar II software 3.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for cluster analysis of DGGE fingerprints. The similarity matrix for the fingerprints was calculated based on the Pearson product-moment correlation coefficient (Pearson, 1926) and the UPGMA (Sneath and Sokal, 1973) was used as clustering algorithm to calculate the dendrogram. The SD of a dendrogram branch was obtained by reconstructing the similarity values from the branch and comparing the values with the original similarity values.

Sequence analysis of DGGE bands

Bands from eubacterial and *Mycobacterium* 16S rRNA gene-based DGGE profiles were excised from the DGGE gel and DNA, eluted at 4°C with 100 μ l of PCR water (Cambrex) for 4 h, was used as template for PCR amplification with the appropriate primers as described above. The PCR products were cloned into the plasmid vector pCR®2.1-TOPO®, using the TOPO TA Cloning kit (Invitrogen, Merelbeke, Belgium) as described by Invitrogen. To analyse

transformants with eubacterial insert, a nested PCR was performed on the clones including a first PCR with M13f and M13r primers as described by Invitrogen and a second PCR with eubacterial primers GC40-63f and 518r on the products of the first PCR as described above. To analyse transformants with *Mycobacterium* insert, PCR was performed on the clones with *Mycobacterium*-specific primers MYCO66f and GC40-MYCO600r as described above, but with an initial denaturation step for 10 min at 94°C. Denaturant gradient gel electrophoresis patterns of cloned fragments were compared with the appropriate fingerprints of the soil eubacterial or *Mycobacterium* community and appropriate clones were chosen for sequence analysis. Polymerase chain reaction products obtained from the clones were purified with the PCR purification kit (Qiagen) as described by Qiagen and subjected to sequencing reactions. Sequencing reactions were performed with the QuickStart DNA sequencing kit (Beckman) using the eubacterial 16S rRNA gene primer 530r (Lane, 1991) and analysed on an automatic sequencer (CEQ™8000, Beckman Coulter, Fullerton, CA, USA). Resulting partial 16S rRNA gene sequences (about 400 bp) were compared with sequences deposited in GenBank by performing a BLASTN search (Altschul *et al.*, 1997).

Nucleotide sequence accession number

The partial 16S rRNA gene sequence data have been submitted to the EMBL database under Accession No. AM085778 to AM085782 and Accession No. AM085785 to AM085788.

Most-probable-number (MPN) estimate of PAH-degrading bacteria in soil and *Mycobacterium* identification by FISH

The MPN enumeration of phenanthrene and pyrene degraders in the different soil fractions was based on the detection of microbial growth on phenanthrene or pyrene in microtitre plates by using the respiration indicator WST-1, as described by Johnsen and colleagues (2002). The MPN enumeration for the different soil fractions was performed after fractionation in duplicate. Bacterial pellets of 10 positive wells of the highest dilutions, resulting from growth on phenanthrene or pyrene, were pooled to have sufficient cells for FISH analysis. *Mycobacterium*-specific FISH analysis was performed by using fluorescently labelled PNA probes (with sequence in parentheses) targeting the 16S rRNA of *M. austroafricanum* (5'-TCTAGGCACGTCCT-3'), *M. frederiksbergense* (5'-GCATGGTCATATTC-3') and *M. gilvum* (5'-ATGCGGTCCTATTC-3'). These probes were specially designed for detection of these three *Mycobacterium* spp. in environmental samples and the applied hybridization conditions will be described elsewhere (B. Joffe and M. Hausner, unpublished). The identification of phenanthrene and pyrene degraders in Table 4 is expressed as a relative percentage of *Mycobacterium* cells (visualized by specific FISH) to eubacterial cells (visualized by DAPI: 4',6-diamidino-2-phenylindole). Bacterial cells were counted in duplicate as an average of 10 microscopic view fields, each containing at least 400 bacterial cells.

Statistical analysis

The Tukey test (SAS® 9.1; Cary, NC, USA) ($P < 0.05$) was used for all statistical comparisons of maximum mineralization rate, lag time and log transformed MPN.

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