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Enrichment and identification of polycyclic aromatic compound-degrading bacteria enriched from sediment samples

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Abstract The degradation of polycyclic aromatic compounds (PACs) has been widely studied. Knowledge of the degradation of PACs by microbial populations can be utilized in the remediation of contaminated sites. To isolate and identify PAC-degrading bacteria for potential use in future bioremediation programmes, we established a series of PAC enrichments under the same experimental conditions from a single sediment sample taken from a highly polluted estuarine site. Enrichment cultures were established using the pollutants: anthracene, phenanthrene and dibenzothiophene as a sole carbon source. The shift in microbial community structure on each of these carbon sources was monitored by analysis of a time series of samples from each culture using 16S rRNA polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Significantly, our findings demonstrate that shifts in the constituent species within each degradative community are directly attributable to enrichment with different PACs. Subsequently, we characterized the microorganisms comprising the degradative communities within each enrichment using 16S rRNA sequence data. Our findings demonstrate that the ability to degrade PACs is present in five divisions of the *Proteobacteria* and *Actinobacteria.* By determining the precise identity of the PAC-degrading bacterial species isolated from a single sediment sample, and by comparing our findings with previously published research, we demonstrate how bacteria with similar PAC degrading capabilities and 16S rRNA signatures are found in similarly polluted environments in geographically very distant locations, e.g., China, Italy, Japan and Hawaii. Such a finding suggests that geographical barriers do not limit the distribution of key PAC-degrading bacteria; this finding is in accordance with the Baas-Becking hypothesis "everything is everywhere; the environment selects" and may have significant consequences for the global distribution of PAC-degrading bacteria and their use in bioremediation.

Keywords Anthracene · Phenanthrene · Dibenzothiophene · PAHs · PACs · Bioremediation

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants with low bioavailability, high persistence in soil and potential deleterious effects on human health (Johnsen et al. 2005). This has been the impetus behind most of the interest in the biodegradation mechanisms and environmental fate of PAHs. On the basis of their abundance and toxicity, 16

PAH compounds have been identified by the US Environmental Protection Agency (EPA) as priority pollutants, including anthracene (ANT) and phenanthrene (PHE) (Habe and Omori 2003).

A vast array of microbial species (bacteria, fungi and algae) can utilize PAHs as sole carbon and energy sources (Van Hamme et al. 2003). The most commonly isolated genus with PAH-degradative capabilities is *Pseudomonas* (Ahn et al. 1999; Cerniglia 1992; Whyte et al. 1997). Other genera that are commonly studied and shown to be PAH-degraders are *Nocardia, Mycobacteria* (Sho et al. 2004; Stingley et al. 2004) and *Sphingomonas* (Cerniglia 1992; Hamann et al. 1999).

Dibenzothiophene (DBT), a polyaromatic sulphur heterocyclic compound, has been found to exist in petroleum, coal, airborne particulates, storm water runoff and in sediments and organisms (Yang and Zhang 1997). Interest into the degradation of DBT arises from its carcinogenic and mutagenic properties combined with their nearly ubiquitous distribution in depositional environments (Yang et al. 1998). DBTs were among the compounds that were most resistant to biodegradation in sediments contaminated with oil from the *Amoco Cadiz* spill (Kropp et al. 1997). The recalcitrance of the DBT contributes to the accumulation of condensed thiophenes in the tissues of shellfish in marine environments that become contaminated with crude oil (Kropp et al. 1997). Polycyclic aromatics compounds (PACs) such as the above mentioned, pose great environmental concern and therefore invoke much interest in the development of strategies to remove PACs from contaminated sites. The pathways for oxidative metabolism of ANT, PHE and DBT have biochemical similarities (Denome et al. 1993) and therefore have been addressed simultaneously in this study.

Bioremediation of contaminated sites relies either on the presence of indigenous degrading bacteria, the capabilities of which might be stimulated in situ, or on the inoculation of selected microorganisms with desired catabolic traits in bioaugmentation techniques (Andreoni et al. 2004). Molecular ecological approaches, combined with traditional laboratory enrichments, have previously been utilised to identify bacterial populations that are functionally important in the biodegradation of organic pollutants (Daane et al. 2001; Launen et al. 2008; Muller et al. 1997; Ni Chadhain et al. 2006).

In this study, our key objective was to isolate and identify PAC-degrading bacteria for use in future bioremediation programmes of contaminated sediments. In order to do this, we established a series of PAC enrichments under the same experimental conditions from a single sediment sample taken from a highly polluted estuarine site. Enrichment cultures were established using the pollutants: ANT, PHE and DBT as a sole carbon source, with the aim of identifying the precise PAC-degrading bacterial species utilising each substrate. By comparison of our data with those from previous studies from different global locations, we anticipate that our findings will help to elucidate the geographical distribution of PAC-degrading bacteria and may have significant consequences for their use in bioremediation.

Materials and methods

Collection of environmental samples and isolation of PAC-degrading bacteria

Samples were collected from the Severn Estuary (Severn Bridge, Bristol, South Gloucestershire, UK). The Bristol Channel and Severn Estuary covers an area of 4,800 km^3 and its tidal range is the second-highest in the world (Joint Nature Conservation Committee 2001). Fossil fuel combustion, urban runoff, shipping, sewage treatment works and various diffuse discharges from industrial areas are all documented as sources of pollutants in the estuary; hydrocarbon compounds (including PAHs) form an important component of such pollutants (Environment Agency, Severn River Basin District Liaison Panel 2008). PAHs associate with sediments due to their low solubility. Values >1 g/l (total PAHs) have been recorded in the Severn Estuary (Royal Haskoning/The Bristol Port Company 2008). The lower reaches of the estuary, from where sediment samples were collected for use within this study, are characterised by oxic conditions (Royal Haskoning/The Bristol Port Company 2008).

Sediment samples were collected for enrichment of PAC-degrading consortia in sterile universal tubes. Three sets (one per substrate) of three replicate cultures were initiated in 50 ml flasks for each time point using 1 g of Severn Bridge sediment and 25 ml minimal salts media (MSM; Tett et al. 1994) spiked

with 200 mg/l of either ANT/PHE/DBT crystals as substrate. Flasks were sealed using Teflon caps and incubated statically for 72 h and then transferred to an orbital shaker at 200 rpm at 25°C. At each time point, samples were removed and separate triplicate cultures were used to monitor growth, DNA extraction and PAH analysis. Absorbance of each culture was measured at 420 nm against an abiotic MSM control to monitor bacterial growth.

PAH analysis

At each time point the enrichment cultures were acidified with H_2SO_4 to pH2 and extracted with dichloromethane (25 ml) overnight in an orbital shaker at 200 rpm. Following extraction the solvent layers were removed and dried with 4 g of anhydrous sodium sulfate (Ni Chadhain et al. 2006). Extracts (2 ml) were analysed on a Varian CP-3800 gas chromatograph with flame ionizing detection using an RTX-5 column. The gas chromatograph program consisted of 6 min at 40°C followed by 10°C per minute increase to 300°C. The concentration of each PAC was calculated by comparison against individual PAC standard curves.

DNA isolation

At each time point, enrichment cultures were centrifuged at 6,800*g* for 10 min. The pellet was resuspended in 0.6 ml cetyl trimethyl ammonium bromide (CTAB) buffer (50:50 of 10% CTAB in 0.7 M NaCl and 240 mM potassium phosphate buffer pH 8.0), then 0.5 ml phenol:chloroform:isoamyl alcohol (pH 8.0) (25:24:1) was added. This was transferred to a screw cap tube (2 ml) containing 0.5–1.0 g of zirconnia/silica beads (0.1 mm diameter) and put into a bead beater machine (Biospec) at 4,300 rpm for 40 s to lyse the cells. The tubes were then centrifuged at 11,337*g* for 5 min. The top aqueous layer was extracted and transferred to a sterile eppendorf tube. The nucleic acids were precipitated by adding 1 ml of 30% polyethylene glycol (PEG)/1.6 M NaCl solution and incubated at room temperature for 24 h. Tubes were centrifuged at 11,337*g* for 10 min, the supernatant was removed and the pellet was washed with ice cold 100% ethanol. The ethanol was removed and the pellet was air dried for 5 min and resuspended in 100 µl ultra pure water (Sigma).

PCR and DGGE analysis

For denaturing gradient gel electrophoresis (DGGE) analysis, GC-clamp primers were used to amplify the 16S rRNA gene from different bacterial species, which correspond to positions 421 and 534 in *Escherichia coli* (Muyzer et al. 1993). All PCR reactions were performed using a ThermoHybaid PCR machine. For each sample being amplified the following was required: 2 µl of each primer (10 pmol/µl), 25 µl master mix (Promega—containing 50 U/ml *Taq* polymerase, 400 µM of each dNTPs, 3 mM $MgCl_2$), 19 µl ultra pure water and 2 µl template DNA (50 ng). In addition a negative control was performed whereby no template DNA was added. Cycling conditions for the 16S rRNA gene were as follows: 95°C 5 min × 1 cycle; 95°C 1 min, 55°C 1 min, 72°C 2 min × 30 cycles; 72°C 5 min × 1 cycle. PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and analyzed by DGGE using a BIORAD system. Samples were run on 8% polyacrylamide gels with a denaturant gradient from 40 to 60%. Electrophoresis was carried out for 18 h at 60 V in 1× TAE buffer at 60°C. Gels were stained by firstly washing for 30 min in a fixing solution (10% ethanol and 0.5% acetic acid), then staining for 20 min in a staining solution (0.1% silver nitrate) and finally washing in a developing solution for 10 min (1.5% sodium hydroxide and 0.8% formaldehyde) with a final wash in fixing solution for 5 min. Gels were photographed under white light using a Auto-ChemiSystem (UVP).

Generation of 16S rRNA clone libraries

Clone libraries of the 16S rRNA gene were constructed from DNA amplified from the Severn Bridge culture after enrichment with ANT, PHE and DBT. PCR amplification was performed using eubacterial primers (PA and PH). PA forward primer: 5′-AGAGT TTGATCCTGGCTCAG-3′ and PH reverse primer: 5′- AAGGAGGTGATCCAGCCGCA-3′ using the PCR cycling conditions described by Edwards et al. (1989). The PCR products were ligated into pGEM-T

Easy Vector (Promega) and transformed into high efficiency competent *E. coli* JM109 cells according to the manufacturer's instructions. The transformed cells were plated on LB plates containing 100 μg/l of ampicillin, 80 μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.5 mM IPTG (isoprobyl-β-D-thiogalactopyranoside) as recommended by the manufacturer. White colonies were selected and checked for inserts. A total of 150 clones were screened using DGGE analysis as described above and 17 clones were selected for further sequence analysis.

Phylogenetic analysis

The sequences obtained from the 16S rRNA sequence analysis were submitted to GenBank for a BLAST search. Additional related sequences were identified from BLAST searches and were retrieved from GenBank. Sequences were aligned using ClustalX (Chenna et al. 2003). Aligned sequences were equivalent to 1,514 bp of 16S rRNA sequence of *Escherichia coli* strain SFC6. Phylogenetic analysis was performed using distance methods and Jukes–Cantor correction (Jukes and Cantor 1969). A neighbour-joining tree was inferred using PAUP 4.0. Bootstrapping was performed on 1,000 resamplings of the alignments.

Results

Bacterial growth and PAC degradation

Enrichment cultures were monitored over time to examine the growth and biodegradation potential of sediment bacterial communities enriched in the presence of ANT, PHE and DBT. Optical density measurements (420 nm) of the enrichments show an increase in biomass over time with a concurrent reduction in the concentration of the growth substrate (Fig. 1). Microbial growth pattern and reduction of PACs are typical of bacteria growing in suspended, shaken cultures with crystalline PAH in amounts exceeding the aqueous solubility as the sole source of energy and carbon (Johnsen et al. 2005). ANT, PHE or DBT were provided as the sole carbon source and therefore microbial growth was attributed to metabolism and utilization of the PACs by the culture. Additionally, a control was established whereby no carbon source was provided; optical density

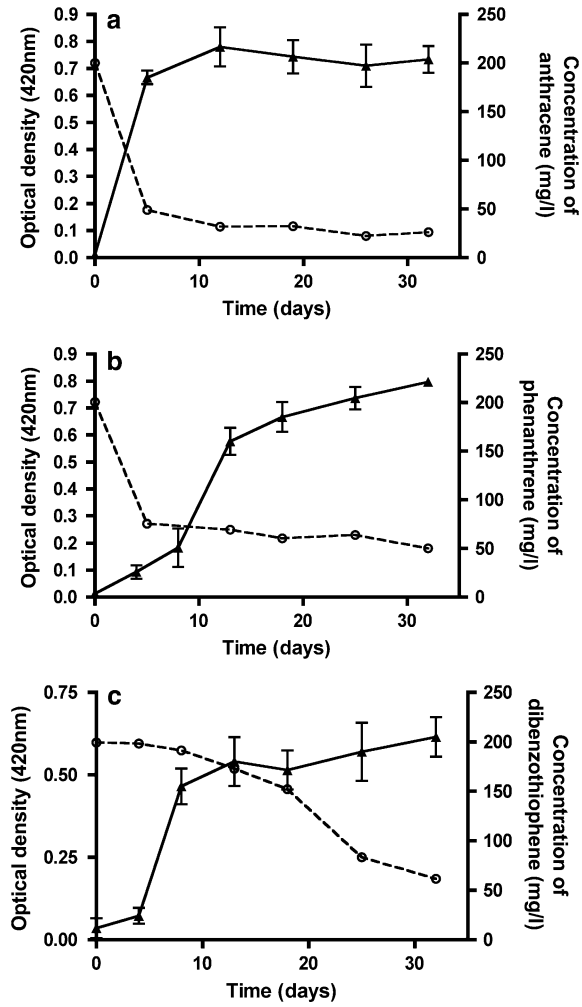


Fig. 1 Microbial growth (▲) and biodegradation of PACs (○) over time. Enrichment culture with anthracene (**a**); enrichment culture with phenanthrene (**b**); enrichment with dibenzothiophene (**c**). Data represents the averages and standard errors of triplicate data

measurements of this culture revealed no microbial growth. ANT degradation by the enrichment culture showed a decrease from 200 to ~50 mg/l by day 5 (Fig. 1a). The ANT enrichment culture showed no lag phase before the onset of exponential growth and reached a maximum optical density of 0.73 (Fig. 1a). PHE degradation by the enrichment culture showed a decrease from 200 to 75 mg/l by day 4 (Fig. 1b). The PHE enrichment culture showed gradual growth over the first 8 days, then exponential growth until day 13 and reached a final optical density of 0.8. DBT degradation occurred at a slower rate, resulting in

a decrease from 200 to 60 mg/l (Fig. 1c). The DBT-degrading culture showed a lag-phase of 4 days and reached a maximum optical density of 0.615.

Bacterial community structure

The effect of ANT/PHE/DBT enrichment on the bacterial community structure was determined throughout the time course using 16S rRNA gene-based PCR-DGGE for each treatment (Fig. 2). The number of DGGE bands was taken as an indication of species in each sample. At time zero the Severn Bridge culture DGGE profile contained numerous bands. Decreases in the number of bands in the ANT/PHE/DBT enrichments were observed by the second time point (5, 4, and 8 days, respectively). DGGE analysis of cultures enriched on individual PACs resulted in unique banding profiles, implying the selection of distinct substrate-specific bacterial populations.

Diversity and species richness of the enrichment libraries

Clone libraries of the 16S rRNA gene were constructed from DNA amplified from the Severn Bridge culture after enrichment with ANT, PHE and DBT. A total of 150 clones were obtained from the Severn Bridge culture when enriched with ANT, PHE and DBT. The clone libraries were screened using DGGE analysis and 17 different clones were selected for complete 16S rRNA sequence analysis. The sequence identity of the selected clones revealed that the ANT enrichment contained two clones identified as *Bradyrhizobium* spp., one clone as *Xanthobacter* sp., one as *Rhizobium* sp., one as *Xanthomonas* sp., one as *Mycoplana* sp. and another as *Pseudomonas* sp. (Table 1). The PHE enrichment contained one clone with closest sequence identity to *Nocardia* sp., one to *Alcaligenes* sp., one to *Achromobacter* sp. and one to

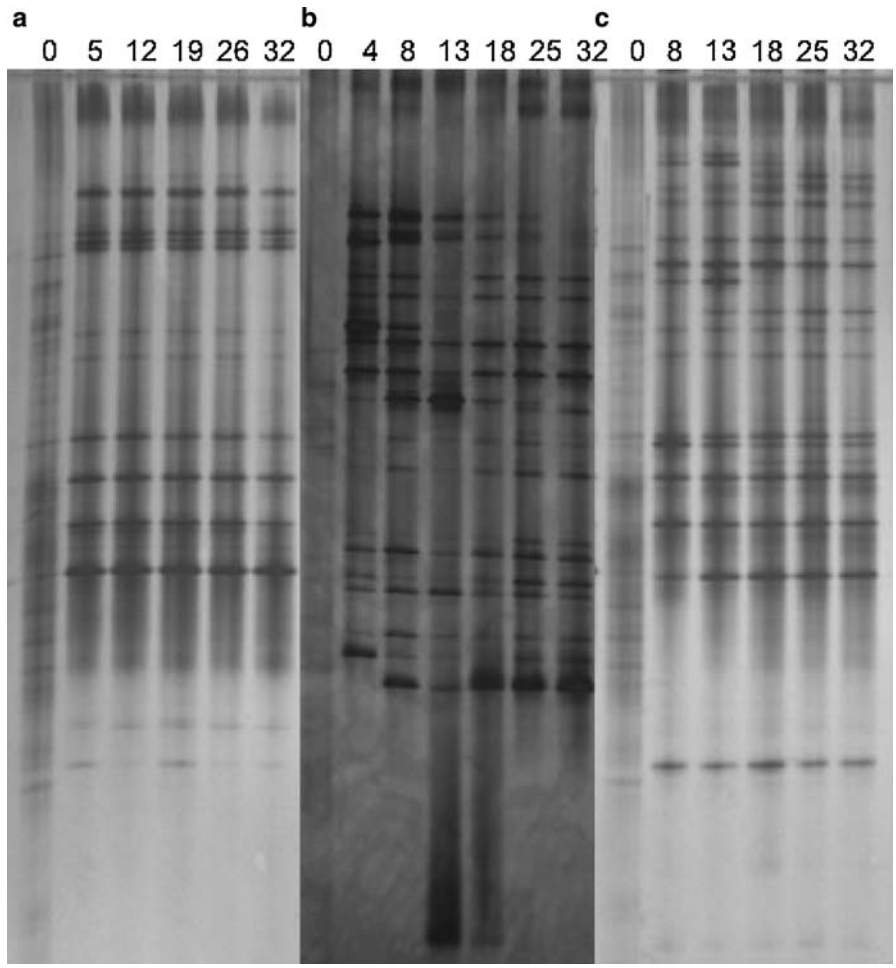


Fig. 2 Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes from anthracene (**a**), phenanthrene (**b**) and dibenzothiophene (**c**) amended enrichment cultures over time. The time of sampling (days) is listed *above* the lanes

Pseudomonas sp. (Table 2). The DBT enrichment contained one clone with the closest sequence identity to an uncultured *β-Proteobacterium* clone, one clone as *Ancylobacter* sp., and one as *Pseudomonas* sp. (Table 3).

Phylogenetic analysis was performed on the ANT, PHE and DBT degradative communities

All 16S rRNA sequences were aligned and a neighbour-joining tree was constructed (Fig. 3). Phylogenetic analysis demonstrated that the 16S rRNA gene clones from the ANT enrichment were in the *α-Proteobacteria* (anthracene clones 2, 12, 14, 33, 41, 46) and *γ-Proteobacteria* (anthracene clones 23, 47). The 16S rRNA gene clones from the PHE enrichment were in the *β-Proteobacteria* (phenanthrene clone 22, 37), *γ-Proteobacteria* (phenanthrene 2, 40) and the *Actinobacteria* (phenanthrene clone 3). The 16S rRNA gene clones from the DBT enrichment were in the *β-Proteobacteria* (dibenzothiophene clones 1, 12), one with the *γ-Proteobacteria* (dibenzothiophene clone 13) and one with the *α-Proteobacteria* (dibenzothiophene clone 3).

Discussion

The ability of bacteria to utilise individual PACs as carbon and energy sources has been well documented

Table 1 16S rRNA clone library of the Severn Bridge culture after 30 days enrichment with anthracene

Clone	Genbank accession no.	Closest organisms in Genbank database	Similarity[a]
2	AJ313028	*Xanthobacter* sp. MN 45.1	0.99
12	DQ917252	Uncultured *Bradyrhizobium* sp. clone 57-8	0.97
14	DQ089696	*Rhizobium daejeonense* strain L22	0.96
23	AP008229	*Xanthomonas oryzae* MAFF 311018	0.97
33	AJ536689	Bacterium RBS4-92	0.99
41	AY238503	*Bradyrhizobium* sp. 1	0.99
46	EU256383	*Mycoplana peli* AN343	0.96
47	AB246809	*Pseudomonas boreopolis*	0.97

[a] Sequences were matched with the closest relative from the Genbank database

Table 2 16S rRNA clone library of the Severn Bridge culture after 30 days enrichment with phenanthrene

Clone	Genbank accession no.	Closest organisms in Genbank database	Similarity[a]
2	DQ297971	Uncultured soil bacterium clone UH3	0.97
3	EF028121	*Norcardiaceae* bacterium Ben-13	0.99
22	AJ509012	*Alcaligenes faecalis* isolate 5659-H	0.99
37	DQ659433	*Achromobacter xylosoxidans* isolate 2MN-2	0.99
40	AJ864722	*Pseudomonas boreopolis* strain S2-s-PMWA-6	0.97

[a] Sequences were matched with the closest relative from the Genbank database

Table 3 Closest genera and percentage of identity of the selected clones isolated from the Severn Bridge culture after 37 days enrichment with dibenzothiophene

Clone	Genbank accession no.	Closest organisms in Genbank database	Similarity[a]
1	AB288556	Uncultured *β* proteobacterium clone RPS-F2	0.99
3	AY056830	*Ancylobacter* sp. AS1.1761	0.97
12	DQ129607	Uncultured bacterium clone AKIW1148	0.99
13	AJ864722	*Pseudomonas boreopolis* strain S2-s-PMWA-6	0.97

[a] Sequences were matched with the closest relative from the Genbank database

(Ahn et al. 1999; Cerniglia 1992; Dean-Ross et al. 2001). In addition, there are numerous studies into the bacterial community dynamics of petroleum-degrading populations (Stoffels et al. 1998; Vinas et al. 2005). The study presented here has investigated the ability of PAC-degrading bacteria within a single sediment culture to degrade three environmentally important pollutants (ANT, PHE, DBT) under the same conditions. We have succeeded in isolating and characterising three distinct, stable PAC-degrading populations, which may be utilised in the subsequent development of bioremediation techniques.

ANT and PHE were degraded at the highest rates (Fig. 1a, b). DBT was degraded at a slower rate (Fig. 1c), which can be explained through the relative complexity of the structure of this compound (Habe and Omori 2003; Omori et al. 1992). Our study allows direct comparison of the degradability of each PAC and indicates that ANT is slightly more readily degraded than PHE, which in turn are both more degradable than DBT. In previous studies of the relative rates of degradation of these three compounds (Hirano et al. 2006; Vinas et al. 2005), the monitoring of degradation of different PACs by single strains or environmental consortia has resulted in differing degradation rates and interpretations of the ease of degradation of each PAC. Uniquely, our study compares the degradation rates of ANT, PHE and DBT by the same bacterial community. Thus, we have been able to assess the recalcitrance of each compound and therefore the degree of associated environmental impact.

The individual effects of each pollutant on the community structure of the Severn Bridge culture were monitored during enrichment by 16S rRNA gene-based PCR-DGGE. At time zero the DGGE profile contained numerous bands, indicating the broad diversity of microbes present in the Severn Bridge culture. Subsequently, a decrease in the number of bands in the profile of each enrichment (ANT, PHE or DBT) was observed (Fig. 2). Accordingly, we suggest that these decreases in diversity over time are most likely attributable to the selection of a limited number of PAC-degrading bacterial species that can utilise each sole carbon source. Enrichment of the Severn Bridge culture with ANT, PHE or DBT established three stable degradative communities. Previously, sourcing microbial strains for bioaugmentation has typically been achieved by selective enrichment and therefore the three stable degradative communities of this study offer potential consortia for bioremediation. DGGE analysis of the cultures enriched on individual PACs resulted in unique banding profiles, implying the selection of distinct substrate-specific bacterial populations, and, due to the experimental approach adopted, the resultant community shifts can be directly attributable to enrichment with different PACs. The distinctive profiles of the degradative communities established emphasize the necessity of utilizing a diverse and complex microbial population when using bioremediation techniques to target sites contaminated with multiple pollutants.

Characterisation of the ANT, PHE and DBT-degradative communities identified three differing populations, the only exception being the clone with highest sequence homology to *P. boreopolis*, which was present in all three populations. *P. boreopolis* has previously been identified as being able to degrade recalcitrant chemosynthetic resin within microorganism communities isolated from compost (Kishimoto et al. unpublished). Similarly, Lloyd-Jones et al. (1999) found that enriching with different substrates, even with closely related PAHs such as phenanthrene and naphthalene, led to the isolation of different genotypic groups. Overall, these findings together with those from our study indicate that assembling a degradative community for bioaugmentation of sites contaminated with multiple PACs will require a complex bioremedial bacterial population.

Phylogenetic analysis of the ANT/PHE/DBT-degrading communities demonstrated that the 16S rRNA gene clones from the ANT/PHE/DBT-enrichments (Fig. 3, shown in boldface) were identified as belonging to the α-, *β*-, *γ*-*Proteobacteria* and the *Actinobacteria*, with the majority belonging to the α-*Proteobacteria*. Degraders reported previously have mostly been identified as either *β*-, *γ*-*Proteobacteria* or as *Actinobacteria*. However, it is important to note that degraders identified previously have been isolated from numerous sampling sites (Muller et al. 1997; Stoffels et al. 1998; Vinas et al. 2005). Such findings serve to highlight the great diversity of microbial species with PAC degrading capabilities that may be exploited in clear-up strategies of contaminated sites. Additionally, characterisation of key PAC-degrading bacteria permits the identification of functionally important microbes and should, therefore, greatly

increase the success of bioremediation techniques, which rely on having the right microbes in the right place with the right environmental factors for degradation to occur (Boopathy 2000). The greater our understanding of the processes that occur and of the microorganisms that are responsible, the more appropriately the techniques can be tailored to site-specific conditions.

There is a great diversity of microbial species that can degrade PACs (Andreoni et al. 2004; Muller et al. 1997; Vinas et al. 2005; Widada et al. 2002) with some genera containing more PAC-degrading species than others. Moreover, it appears that the physiological conditions at both the site of origin of a PAC-degrading population and the conditions used to isolate a population will affect the final composition of a remediation community. Certainly, the results of this study, which utilised a single sediment culture to establish ANT-, PHE- and DBT-degrading populations, whilst keeping all other variables constant, have demonstrated that the specific petroleum hydrocarbon substrate used in isolation affect the species present in the final degradative community.

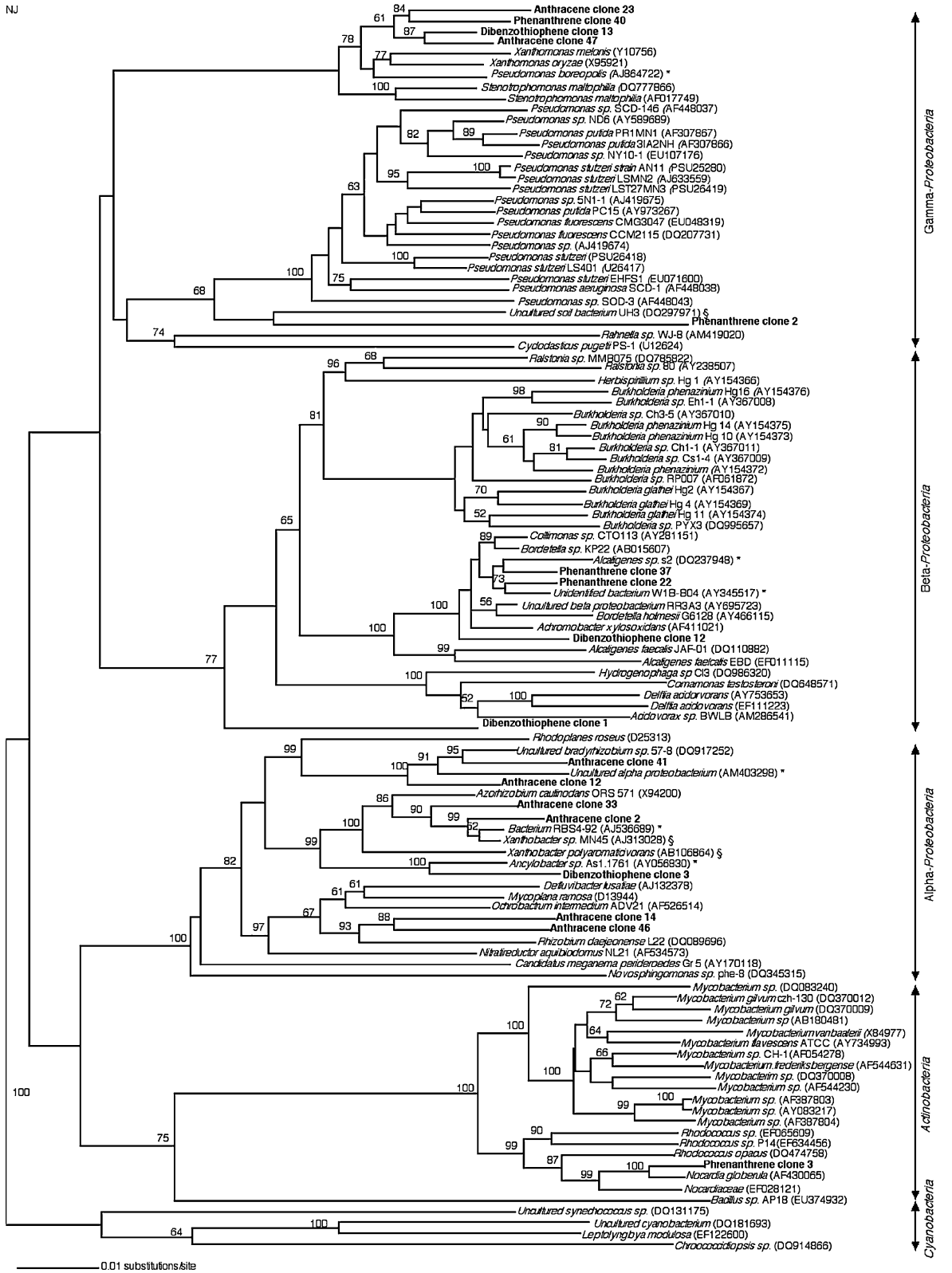
Characterisation of the ANT-, PHE- and DBT-degrading bacteria using phylogenetic methods clustered the PAC-degrading clones with three of the five divisions of *Proteobacteria* (α, β and γ) and to *Actinobacteria* (Fig. 3). This analysis reveals the diversity present within the initial sediment sample and within the stable degradative cultures. Furthermore, it indicates the extent to which PAC-degradative capabilities have spread across bacterial taxonomic divisions. Our results, which may indicate that the ability to utilise different substrates is due to horizontal gene transfer (HGT) rather than independent evolution of such traits, is in accord with previous research into HGT of PAC-degradative genes (Herrick et al. 1997; Johnsen et al. 2005; Wilson et al. 2003).

Comparison of the PAC-degrading species enriched from single sediment samples in our study with previously published research revealed that bacteria with similar PAC-degrading capabilities and 16S rRNA signatures can be found in similarly polluted environments in geographically distant locations e.g., China, Italy, Japan and Hawaii (Fig. 3; taxa marked with *). Other studies have found a correlation between bacterial composition and environmental or geographic characteristics, such as salinity, depth and latitude (Cho and Tiedje 2000; Crump et al. 2004; Hughes Martiny et al. 2006). PAC-degrading species isolated from the Severn Estuary sediment were found to have similar 16S rRNA signatures to species isolated from hydrocarbon-contaminated sites (Fig. 3, taxa marked with §) e.g., *Xanthobacter* sp. (AJ313028) isolated from hexane-degrading biofilters, *Xanthobacter polyaromaticivorans* (AB106864) isolated from anoxic crude oil tank sludge, and an uncultured soil bacterium (DQ297971) isolated from hydrocarbon-contaminated soil. Overall, such findings suggests that geographical barriers do not limit the distribution of PAC-degrading bacteria when an appropriate hydrocarbon substrate is present, a finding in accordance with the Baas-Becking hypothesis "everything is everywhere, the environment selects" (Baas-Becking 1934).

Conversely, however, this study has also isolated clones which have high 16S rRNA sequence homology with species isolated from seemingly unassociated sites, e.g., a cyanide treatment bioreactor in Hawaii (unidentified bacterium W1B-B04, AY345517; see Fig. 3 within the *β-Proteobacteria*), a very different environment from those recreated here. The significance of such a result remains to be explored.

Overall, the findings of this study emphasize the global distribution and diversity of PAC-degrading bacteria, an important factor when sourcing microbial strains for bioaugmentation and developing

Fig. 3 Neighbour-joining tree constructed using PAUP 4.0 ▶ showing the phylogenetic relationship of 16S rRNA sequences cloned from the Severn Bridge culture when enriched on anthracene (eight clones), phenanthrene (five clones) and dibenzothiophene (four clones) to 94 reported sequences including PAC-degrading bacteria (GenBank). Bootstrap percentages of 50% or more are indicated. *Scale bar* indicates Jukes–Cantor distances. The α-, β- and γ-subclasses of the *proteobacteria*, the *actinobacteria* and *cyanobacteria* groups have been indicated. The clones of this study are shown in boldface. Taxa marked with * denote species isolated from geographically distinct locations from those isolated in this study: *Pseudomonas boreopolis* (AJ862722) from Japan, *Alcaligenes* sp. s2 (DQ237948) from China, unidentified bacterium W1B-B04 (AY345517) from Hawaii, uncultured alpha *proteobacterium* (AM403298) from Germany and bacterium RBS4-92 (AJ536689) from Italy. Taxa marked with § denote species isolated from locations where hydrocarbons are present: uncultured soil bacterium UH3 (DQ297971) from hydrocarbon-contaminated site, *Xanthobacter* sp. MN45 (AJ313028) from hexane-degrading biofilters and *Xanthobacter polyaromaticivorans* (AB106864) from anoxic crude oil tank sludge


NJ
Anthracene clone 23
Phenanthrene clone 40
Dibenzothiophene clone 13
Anthracene clone 47
Xanthomonas melonis (Y10756)
Xanthomonas oryzae (X95921)
Pseudomonas boreopolis (AJ864722) *
Stenotrophomonas maltophilia (DQ777866)
Stenotrophomonas maltophilia (AF017749)
Pseudomonas sp. SCD-146 (AF448037)
Pseudomonas sp. ND6 (AY589689)
Pseudomonas putida PR1MN1 (AF307867)
Pseudomonas putida 3IA2NH (AF307866)
Pseudomonas sp. NY10-1 (EU107176)
Pseudomonas stutzeri strain AN11 (PSU25280)
Pseudomonas stutzeri LSMN2 (AJ633559)
Pseudomonas stutzeri LST27MN3 (PSU26419)
Pseudomonas sp. 5N1-1 (AJ419675)
Pseudomonas putida PC15 (AY973267)
Pseudomonas fluorescens CMG3047 (EU048319)
Pseudomonas fluorescens CCM2115 (DQ207731)
Pseudomonas sp. (AJ419674)
Pseudomonas stutzeri (PSU26418)
Pseudomonas stutzeri LS401 (U26417)
Pseudomonas stutzeri EHFS1 (EU071600)
Pseudomonas aeruginosa SCD-1 (AF448038)
Pseudomonas sp. SOD-3 (AF448043)
Uncultured soil bacterium UH3 (DQ297971) §
Phenanthrene clone 2
Rahnella sp. WJ-8 (AM419020)
Cytodadstous pugetii PS-1 (U12624)
Ralstonia sp. MMB075 (DQ785822)
Ralstonia sp. 80 (AY239507)
Herbispirillum sp. Hg 1 (AY154366)
Burkholderia phenazinium Hg16 (AY154376)
Burkholderia sp. Eh1-1 (AY367008)
Burkholderia sp. Ch3-5 (AY367010)
Burkholderia phenazinium Hg 14 (AY154375)
Burkholderia phenazinium Hg 10 (AY154373)
Burkholderia sp. Ch1-1 (AY367011)
Burkholderia sp. Cs1-4 (AY367009)
Burkholderia phenazinium (AY154372)
Burkholderia sp. RP007 (AF061872)
Burkholderia glathei Hg2 (AY154367)
Burkholderia glathei Hg 4 (AY154369)
Burkholderia glathei Hg 11 (AY154374)
Burkholderia sp. PYX3 (DQ995657)
Collimonas sp. CTO113 (AY281151)
Bordetella sp. KP22 (AB015607)
Alcaligenes sp. s2 (DQ237948) *
Phenanthrene clone 37
Phenanthrene clone 22
Unidentified bacterium W1B-B04 (AY345517) *
Uncultured beta proteobacterium RR3A3 (AY695723)
Bordetella holmesii G6128 (AY466115)
Achromobacter xylosoxidans (AF411021)
Dibenzothiophene clone 12
Alcaligenes faecalis JAF-01 (DQ110882)
Alcaligenes faecalis EBD (EF011115)
Hydrogenophaga sp CI3 (DQ986320)
Comamonas testosteroni (DQ648571)
Delftia addorvorans (AY753653)
Delftia addovorans (EF111223)
Acidovorax sp. BWLB (AM286541)
Dibenzothiophene clone 1
Rhodoplanes roseus (D25313)
Uncultured bradyrhizobium sp. 57-8 (DQ917252)
Anthracene clone 41
Uncultured alpha proteobacterium (AM403298) *
Anthracene clone 12
Azorhizobium caulinodans ORS 571 (X94200)
Anthracene clone 33
Anthracene clone 2
Bacterium RBS4-92 (AJ536689) *
Xanthobacter sp. MN45 (AJ313028) §
Xanthobacter polyaromaticivorans (AB106864) §
Ancylobacter sp. As1.1761 (AY056930) *
Dibenzothiophene clone 3
Defluvibacter lusatiae (AJ132378)
Mycoplana ramosa (D13944)
Ochrobactrum intermedium ADV21 (AF526514)
Anthracene clone 14
Anthracene clone 46
Rhizobium daejeonense L22 (DQ089696)
Nitratireductor aquibiodomus NL21 (AF534573)
Candidatus meganema perideroedes Gr 5 (AY170118)
Novosphingomonas sp. phe-8 (DQ345315)
Mycobacterium sp. (DQ083240)
Mycobacterium gilvum czh-130 (DQ370012)
Mycobacterium gilvum (DQ370009)
Mycobacterium sp (AB180481)
Mycobacterium vanbaalenii (X84977)
Mycobacterium flavescens ATCC (AY734993)
Mycobacterium sp. CH-1(AF054278)
Mycobacterium frederiksbergense (AF544631)
Mycobacterim sp. (DQ370008)
Mycobacterium sp. (AF544230)
Mycobacterium sp. (AF387803)
Mycobacterium sp. (AY083217)
Mycobacterium sp. (AF387804)
Rhodococcus sp. (EF065609)
Rhodococcus sp. P14(EF634456)
Rhodococcus opacus (DQ474758)
Phenanthrene clone 3
Nocardia globerula (AF430065)
Nocardiaceae (EF028121)
Bacillus sp. AP18 (EU374932)
Uncultured synechococcus sp. (DQ131175)
Uncultured cyanobacterium (DQ181693)
Leptolyngbya modulosa (EF122600)
Chroococcidiopsis sp. (DQ914866)
Gamma-Proteobacteria
Beta-Proteobacteria
Alpha-Proteobacteria
Actinobacteria
Cyanobacteria
0.01 substitutions/site

bioremediation strategies. In particular, our findings confirm the ability of microorganisms to adapt to the environment via their ability to transfer functional genes, high densities and shorter generation times, allowing them to undergo rapid genetic divergence (Hughes Martiny et al. 2006). This would explain the wide distribution of PAC-degradative capabilities, but also why PAC-degraders identified in this study have clustered with functionally unrelated species from distant locations. These findings affirm the adaptability of microbes and their great potential to be exploited for clearing-up hydrocarbon contaminated sites, either through inoculation of specifically isolated microbial communities or through targeted stimulation of the selected species in situ.

Conclusion

The bioremediation of polluted sites is urgent because many PAHs are toxic, carcinogenic or mutagenic. By keeping all experimental conditions constant, this study has established the effect each PAC has on the community structure originating from a single sediment sample, and has identified three stable degradative communities, containing diverse bacterial species that are potential candidates for bioremediation. Comparison of the PAC-degraders identified in this study (α-, β-, γ-*Proteobacteria* and *Actinobacteria*) with those from previously published research has demonstrated that bacteria with similar PAC-degrading capabilities and 16S rRNA signatures are found in similarly polluted environments in geographically very distant locations. This implies that not only has the ability to degrade PACs spread across phylogenetic divisions, but that microbes have physically spread, unhindered by geographical barriers. The disparity of the three degradative communities studied highlights the considerations necessary when sourcing strains for bioaugmentation of sites contaminated with multiple PACs. The empirical approach adopted in many bioremediation processes may be due to the limited understanding of biodegradative systems and microbial populations—the experimental approach of this study, allowing direct comparison of the effect of each PAC on a single bacterial culture and characterisation of functionally important microbes, greatly increases the chance of success of future bioremediation techniques.

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