THE DISTRIBUTION AND DIVERSITY OF POLYCYCLIC AROMATIC COMPOUND-DEGRADING BACTERIA AND KEY DEGRADATIVE GENES

Submitted by

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Abstract

Petroleum hydrocarbons are the most widespread contaminants in the environment. Interest in the biodegradation of polycyclic aromatic hydrocarbons and compounds (PAHs/PACs) is motivated by their ubiquitous distribution, their low bioavailability, high persistence in soils and their potentially deleterious effects to human health. Identifying the diversity of microorganisms that degrade PAHs/PACs can be utilised in the development of bioremediation techniques. Understanding the mechanisms of bacterial populations to adapt to the presence of pollutants and the extent that lateral transfer of key functional genes occurs, will allow the exploitation of microbial PAC/PAH-degradative capabilities and therefore enhance the successful application of bioremediation strategies.

A key aim of this study was to isolate and identify PAC-degrading bacteria for potential use in future bioremediation programmes. A series of PAC enrichments were established under the same experimental conditions from a single sediment sample taken from a highly polluted estuarine site. Distinct microbial community shifts were directly attributable to enrichment with different PAC substrates. The findings of this study demonstrate that five divisions of the *Proteobacteria* and *Actinobacteria* can degrade PACs. By determining the precise identity of the PAC-degrading bacteria isolated, and by comparing these with previously published research, this study showed 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 is significant when considering the diversity and global distribution of microbes with PAC-degradative capabilities and the potential for utilising these microbial populations in future bioremediation strategies.

In the laboratory, enrichment of bacteria able to utilise PAHs has commonly been performed in liquid media, with the PAH dissolved in a carrier solvent. This study found the presence of a carrier solvent significantly affects the resultant microbial population. Although the same sediment sample was used as the bacterial source in all enrichments, different bacterial strains were obtained depending upon the presence of the carrier solvent and the PAH. This is important when considering appropriate methodology for the isolation of PAH-degrading bacteria for future bioremediation programmes. Additionally, the species comprising the resultant population of the enrichment when a carrier solvent was present were similar to previously reported PAH-degrading species. Such a finding necessitates review of previously reported PAH-degrading bacterial species that have been isolated and identified from enrichments using a carrier solvent.

Understanding how bacteria acclimatise to environmental pollutants is vital for exploiting these mechanisms within clear up strategies of contaminated sites. Two major lineages of the α subunit of PAH dioxygenases were identified: *Actinobacteria* and *Proteobacteria.* Comparison of the α subunit phylogeny with the 16S rRNA phylogeny implies that the PAH-dioxygenases evolved prior to the separation of these phyla or that lateral transfer occurred in the very distant past. No evidence for lateral transfer of the α subunit between the *Actinobacteria* and *Proteobacteria* was found in the phylogenetic analyses of this research. Multiple lateral transfer events were inferred between the species of the *Actinobacteria* and between the classes of the *Proteobacteria*. The clustering of the taxa within the α subunit phylogeny indicates that lateral transfer of the α subunit gene occurred after the separation of the classes of *Proteobacteria* and also after the speciation of the γ-*Proteobacteria.* These findings reveal how bacteria have acclimatised to PAH pollutants through multiple lateral transfer events of a key PAH-degradative gene. This knowledge of the transfer of genetic material will broaden our prospects of exploiting microbial populations.

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Abbreviations

ANT	Anthracene
API gravity	American Petroleum Institute gravity
CTAB	Ceylrimethylammonium bromide
DBT	Dibenzothiophene
DGGE	Denaturing gradient gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
GC-FID	Gas chromatography-Flame ionisation detector
HGT	Horizontal gene transfer
ISP	Iron-sulphur protein
HMW PAHs	High molecular weight PAHs
LMW PAHs	Low molecular weight PAHs
MGE	Mobile genetic element
MSM	Minimal salts medium
nahAc/ndoB/nidA	Encodes the large iron-sulphur (Fe-S) subunit of naphthalene dioxygenase
NDO	Naphthalene dioxygenase
PAC	Polycyclic aromatic compound
PAH	Polycyclic aromatic hydrocarbon
pahAc	Encodes the large Fe-S subunit of phenanthrene dioxygenase
PCR	Polymerase chain reaction
PEG	Poly ethylene Glycol
PHE	Phenanthrene
phnAc	Encodes the large Fe-S subunit of phenanthrene dioxygenase
*Pp*9816-4	*Pseudomonas putida* strain 9816-4
*Pp*G7	*Pseudomonas putida* strain G7
*Pp*OUS82	*Pseudomonas putida* strain OUS82
RBF	Round bottom flask
TAE	Tris-acetate-EDTA

CHAPTER ONE

INTRODUCTION

1 Introduction

1.1 The fate of oil in natural environments

1.1.1 General introduction

Oil and oil products are vital to modern society and vast quantities are consumed each year. It is therefore unsurprising that petroleum hydrocarbons are the most widespread contaminants in the environment (Margesin *et al*, 2003). On average, more than 10^8 tonnes of crude oil are shipped every month in huge tankers, a few of which can hold as much as 500,000 tons. This magnitude of oil transportation poses a risk of accidental oil spills (United Nations Environment Programme report no50, Institute of Petroleum). It has been estimated that worldwide, somewhere between 1.7 and 8.8 x 10^6 tons of petroleum hydrocarbons impact marine waters and estuaries annually (Head and Swannell, 1999). Large oil spills, such as the *Exxon Valdez* and *Sea Empress* incidents, invariably capture media coverage but actually contribute little to polluting the environment because such events are relatively rare. However, a substantial number of smaller releases of petroleum hydrocarbons occur regularly in coastal waters. Around the coast of the UK alone, between 1986 and 1996, 6,845 oil spills were reported. Of these, 1,497 occurred in environmentally sensitive areas or were of sufficient magnitude to require clean up (Head and Swannell, 1993).

There are three potential sources from which petroleum and its products can enter the soil environment and possibly reach underground water. The greatest contributor is continuous low-level input, for example, from road surfaces, domestic waste and low-level seepage from oil underground storage tanks. Major spillages from tankers, pipelines and storage tanks are a relatively minor contributor to overall pollution levels. The final source of petroleum hydrocarbon pollution is from slow natural seepage from oil reservoirs (Morgan and Watkinson, 1989). It was reported in 1993 that approximately 50% of petroleum hydrocarbons that affect marine environments originate from industrial effluent and urban/river run off, 19% from shipping operations, 11% natural sources and 5% from tanker accidents (United Nations Environment Programme report no50, Institute of Petroleum).

Oil is a natural substance and therefore under favourable conditions it can disperse

and eventually be degraded through natural processes. However, crude oils and petroleum products are complex substances and their different chemical compounds can react within the environment in a variety of ways.

Damage to ecosystems by oil has been recorded in many parts of the world. It is particularly acute in sensitive intertidal communities where oil spills accumulate over time and have a major impact on sublittoral benthic plant communities important to fishery spawning and recruitment areas (Braddock *et al*, 1995). Oil from the *Braer* spill posed a considerable threat to the coastal salmon farming industry, though fortunately the oil has not persisted on the Shetland shorelines (Swannell *et al*, 1996). Some crude oils leave sticky residues as they weather, which may either float on the surface until they reach the shore, smothering animals and seaweeds, or form solid balls of tar (United Nations Environment Programme report no50, Institute of Petroleum). The *Exxon-Valdez* oil spill killed more wildlife than any prior spill: an estimated 100,000-300,000 sea birds, including hundreds of bald eagles, and thousands of marine mammals.

Investigations after the *Exxon Valdez* oil spill showed that several vertebrate predators experienced physiological stress related to oil toxicity (Bodkin *et al*, 2002). For example, oiled sea otters suffered emphysema, ulcers, anaemia, lesions and organ congestion, whilst river otters showed lower body mass, elevated values of biomarkers, the selection of different habitats with larger home ranges and had less diverse diets (Ben-David *et al*, 2001). Exposure to oil after the *Exxon Valdez* spill affected pacific herring embryo development, resulting in gross malformations, genetic damage and reduced survival from egg to hatching (Hose *et al,* 1994). Studies after the *Exxon Valdez* oil spill demonstrated that fish embryos exposed to low levels of polycyclic aromatic hydrocarbons (PAHs) in weathered crude oil develop a syndrome of oedema and craniofacial and body axis defects (Incardona *et al*, 2005).

Between 8-16% of the 39,000 metric tons of crude oil spilled by *Exxon Valdez* remains buried in marine sediments and therefore poses a continuing threat. Oil buried in sediment is not subjected to aerobic degradation and therefore remains toxic to many vertebrates yet could be re-suspended during storms or tidal activity posing further risk of affecting the ecosystem (Ben-David *et al*, 2001).

1.1.2 Chemistry of oil and hydrocarbons

Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallo constituents, most noticeably vanadium and nickel (Van Hamme *et al*, 2003). Petroleum hydrocarbons can be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and resins (pyridines, quinolines, carbazoles, sulfoxides and amides) (Leahy and Colwell, 1990).

1.2 Biodegradation of hydrocarbons

Laboratory degradation experiments and monitoring of surface oil seeps have revealed compositional changes of petroleum (e.g. sequential and systematic removal of various hydrocarbons; selective degradation of specific isomers and production of acidic compounds) that are distinct from the alteration caused by physical processes (e.g. water washing or phase fractionation) and suggest biological alteration of oils *in situ* (Head *et al*, 2003). Hydrocarbons differ in their susceptibility to microbial attack whereby lighter oils degrade more quickly than heavier oils and *n*-alkanes degrade more rapidly than branched alkanes (Wang *et al*, 1998). Hydrocarbons within the saturated fraction include *n*-alkanes, branched alkanes and cycloalkanes (naphthenes). In a petroleum mixture the *n*-alkanes are considered the most readily degraded component whilst cycloalkanes are the most resistant to microbial attack (Atlas, 1981).

The effects of biodegradation on the composition and physical properties of crude oil and natural gas are well known. Biodegradation of oil results in a decrease in saturated and aromatic hydrocarbon content, whilst there is an increase in oil density, sulphur content, acidity and viscosity (Roling *et al*, 2003). This will obviously have significant negative economic consequences for oil production and refining operations. During the biodegradation of petroleum, hydrocarbons are preferentially degraded but sulphur-, oxygen- and nitrogen-containing compounds can also be degraded. New compounds, such as acyclic and cyclic, saturated and aromatic carboxylic acids and phenols are produced from hydrocarbons, and a complex variety of acidic non-hydrocarbons are generated from the aromatic heterocycles found in oil

(Head *et al*, 2003).

1.2.1 Physical and chemical factors affecting the biodegradation of hydrocarbons

The persistence of petroleum pollutants depends on the quantity and quality of the hydrocarbon mixtures and on the properties of the affected ecosystem. In one environment petroleum hydrocarbons can persist indefinitely, whereas under another set of conditions the same hydrocarbons can be completely degraded within relatively few hours or days. For example, it was found that *n*-alkanes within Venezuelan crude oil were degraded less than the same *n*-alkanes within an Arabian crude oil (Mulkins-Phillips and Stewart, 1974).

1.2.1.1 Physical state of oil pollutants

Oil spilled in water tends to spread and form a slick. As a result of wind and wave action, oil-in-water or water-in-oil ("mousse") emulsions may form (Cooney, 1984). Dispersion of hydrocarbons in the water column in the form of oil-in-water emulsions increases the surface area of the oil and thus its availability for microbial attack, however, large masses of mousse establish unfavourably low surface-to-volume ratios, inhibiting biodegradation (Leahy and Colwell, 1990). Hydrocarbon-degrading bacteria act mainly at the oil-water interface and can be observed growing over the entire surface of an oil droplet (Atlas, 1981). Tarballs also restrict access by microorganisms because of their limited surface area. Biosurfactants reduce surface tension, critical micelle concentration and interfacial tension in both aqueous solutions and hydrocarbon mixtures and are important in the uptake of hydrocarbons by bacteria (Banat, 1995).

The key differences between petroleum biodegradation in soil and aquatic ecosystems following an oil spill are related to the movement and distribution of the oil and the presence of particulate matter, each of which affects the physical and chemical nature of the oil and hence its susceptibility to microbial attack (Bossert and Bartha, 1984). Terrestrial oil spills are characterised primarily by the vertical movement of the oil into the soil, rather than the horizontal spreading associated with slick formation. Infiltration of the oil into the soil prevents evaporative losses of volatile

hydrocarbons, which can be toxic to microorganisms. Particulate matter can reduce, by absorption, the effective toxicity of the components of petroleum, but absorption and adsorption of hydrocarbons to humic substances probably contribute to the formation of persistent residues (Calvalca *et al*, 2008).

1.2.1.2 Concentration of the oil or hydrocarbons

High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, causing inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons (Fusey and Oudot, 1984). The rates of mineralization of the higher-molecular-weight aromatic hydrocarbons, such as naphthalene and phenanthrene, are related to aqueous solubilities rather than total substrate concentrations (Johnsen *et al*, 2005). The concept of maximum or threshold concentrations for microbial degradation of hydrocarbons may apply also to soil ecosystems.

1.2.1.3 Temperature

Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community. At low temperatures, the viscosity of the oil increases, the volatilization of toxic short-chain alkanes is reduced and their water solubility is increased, delaying the onset of biodegradation. Despite this, a large number of degrading bacteria from contaminated cold soils have been identified, including representatives of Gram-negative and Gram-positive genera (Margesin *et al*, 2003). Rates of degradation are generally observed to decrease with decreasing temperature; this is believed to be a result primarily of decreased rates of enzymatic activity (Leahy and Colwell, 1990). Increasing the temperature from 30 to 40ºC increases the rates of hydrocarbon metabolism to a maximum, however, above these temperatures the membrane toxicity of hydrocarbons is increased (Leahy and Colwell, 1990). Soil in mesocosm studies of sub-Antarctic soil artificially contaminated with diesel or crude oil responded positively to a temperature increase from 4˚C to 20˚C (Coulon *et al*, 2005).

Similarly, it appears that too great a temperature will inhibit the degradation of hydrocarbons. However, degradation of petroleum occurs within reservoirs up to

temperatures of 80-90°C and hyperthermophilic microorganisms have been successfully isolated from reservoirs of this temperature (Roling *et al*, 2003).

1.2.1.4 Oxygen

Catabolism of aliphatic, cyclic and aromatic hydrocarbons by microorganisms involves the initial oxidation of the substrate by dioxygenase incorporating oxygen from molecular oxygen (Cerniglia, 1992). Aerobic conditions are therefore required for the oxidation of hydrocarbons and are generally found in the upper levels of the water column in marine and fresh water environments. However, oxygen may be limited within aquatic sediments (Fritzche, 1994). Oxygen availability can vary within soil depending on the rate of microbial oxygen consumption, the type of soil and whether the soil is waterlogged. Oxygen has been identified as the limiting factor of the biodegradation of petroleum in soil and gasoline in groundwater (Jameson *et al*, 1975). Anaerobic degradation of petroleum hydrocarbons by microbes has been shown to occur (Coates *et al*, 1997; Meckenstock *et al*, 2004). Anaerobic degradation of PAHs has been demonstrated in several microcosm studies with nitrate, ferric iron or sulphate as electron acceptors and under methanogenic conditions (Meckenstock *et al*, 2004).

1.2.1.5 Nutrients

Hydrocarbons released into aquatic environments, which contain low concentrations of inorganic nutrients, often produces excessively high carbon/nitrogen and/or carbon/phosphorus ratios, which are unfavourable for microbial growth (Atlas, 1981). Adjustment of carbon/nitrogen/phosphorus ratios by the addition of nitrogen and phosphorus in the form of oleophilic fertilizers stimulates the biodegradation of crude oil and individual hydrocarbons in seawater and soil (Coulon *et al*, 2005; Horowitz and Atlas, 1977).

1.2.1.6 Salinity

The effect of salinity on the degradation of hydrocarbons appears to be complex. Kastner *et al* (1998) investigated the impact of salinity on the degradation of PAHs and survival of PAH-degrading bacteria introduced into soil. Salinity repressed the PAH degradation activity of the introduced bacteria and the indigenous soil microflora (Kastner *et al*, 1998). Mille *et al* (1991) investigated the biodegradation of

crude oil by a mixed bacterial community isolated from a marine sediment in varying concentrations of sodium chloride. The amount of oil degraded increased initially with increasing salt concentration to a maximum level at 0.4 mol/l NaCl. Thereafter the amount of oil degraded decreased with increasing salt concentrations (Mille *et al*, 1991). The effect of varying salinity on phenanthrene mineralisation was examined in sediment along a natural salinity gradient in an urban tidal river (Shiaris, 1989). Rates of phenanthrene mineralization related significantly with increasing salinity. Rates ranged from 1 ng/hour/g dry sediment at the freshwater site to >16 ng/hour/g dry sediment at the 30% salinity site. Further investigation led the authors to conclude that phenanthrene degraders in low salinity estuarine sediments subject to salt water intrusion are tolerant to a wide range of salinities but phenanthrene degradation in brackish waters is mainly a function of obligate marine microorganisms (Shiaris, 1989).

1.2.2 Biological factors affecting the biodegradation of hydrocarbons

Hydrocarbons in the environment are biodegraded primarily by bacteria and fungi. Individual organisms can metabolise only a limited range of hydrocarbon substrates, but mixed assemblages of microbes display broad enzymatic capacities that are capable of degrading complex mixtures of hydrocarbons such as crude oil and high molecular weight (HMW) PAHs in soils, freshwater and marine environments (Habe and Omori, 2003). Lower molecular weight PAHs, such as naphthalene and phenanthrene, are degraded most rapidly in sediments, whereas higher molecular weight PAHs, such as benz[a]anthracene, chrysene or benzo[a]pyrene, are quite resistant to microbial attack (Cerniglia, 1992).

1.3 Genetic control of the degradation of environmental pollutants

1.3.1 Introduction

A broad definition of xenobiotics includes all compounds that are released in any compartment of the environment by the action of man and thereby occur at a concentration that is higher than natural (Top and Springael, 2003). Many environmental pollutants such as heavy metals PAHs and oil derivatives (e.g. toluene) are included in this definition. There are several mechanisms by which microbial populations can adapt to the presence of xenobiotics. Firstly, there can be an increase

in the populations of the organisms that already possess catabolic genes that enable them to utilize the pollutant. Secondly, cells can adapt through mutations, such as single nucleotide changes and DNA rearrangements, that result in resistance to or degradation of the compound. Thirdly, cells may acquire genetic information from related or phylogenetically distinct populations in the community by horizontal gene transfer (HGT). Eventually, the individual cells within the population that are best able to resist or degrade the xenobiotic will have a selective advantage and will proliferate until they constitute a majority of the microbial community (Top and Springael, 2003).

1.3.2 Horizontal transfer of functional genes between bacteria

Microbial communities possess a dynamic gene pool, and novel genetic combinations that arise due to horizontal gene transfer (HGT) act as a driving force to shape genomes (Barkay and Smets, 2005). With the exchange of existing genetic material between microorganisms, different DNA fragments can be combined together in new hosts and can either be incorporated or recombined, with the effect that new mosaic genetic structures are created. Upon successful expression of the new genetic mosaic, a metabolic pathway composed of parts previously existent in different organisms can now appear within one individual. This may engender a strong growth advantage when the specific carbon source for the metabolic pathway is present (van der Meer and Sentchilo, 2003).

Comparing microbial genomes for evidence of HGT is a retrospective approach, detecting such events based on changes that HGT make on DNA sequences and on gene distributions, deletions and insertions. When supported by robust statistical analyses, the presence of a conflicting phylogeny for specific genes and a consensus phylogeny based on the majority of the genes, suggests inheritance of the former by HGT (Barkay and Smets, 2005). Mobile genetic elements (MGEs) can be exchanged promiscuously between a broad spectrum of bacteria and contribute to bacterial genome plasticity and therefore contribute significantly to horizontal gene transfer. MGEs include insertion sequences, transposons, integrons, bacteriophages, plasmids and combinations of these elements (Sorensen *et al*, 2005). Genomic islands are a wide range of large mobile (or potentially mobile) defined DNA segments that are

found integrated into the chromosome or other replicons (Springael and Top, 2004).

The role of MGEs in the distribution of catabolic pathways and in the adaptation of a microbial community to a pollutant stress has recently been shown using microcosms, where donor bacteria carrying a catabolic MGE were introduced into an environment that was subsequently challenged with the xenobiotic specific for that MGE. In many cases, transfer of the MGE to members of the indigenous community was shown. In some cases, this was accompanied by community changes and enhanced degradation of the xenobiotic by the 'adapted' microbial community (Springael and Top, 2004). It is evident that the presence of MGEs and the occurrence of HGT facilitate the evolution of bacterial populations to cope with oil and oil components.

The genomes of several microbes relevant to biodegradation have been published (e.g. naphthalene degradation by *Pseudomonas putida* G7 (Simon *et al*, 1993). This information provides an opportunity to gain insights into the potential of specific microorganisms to bioremediate polluted environments and might allow them to be exploited for oil recovery. This will be discussed in the applications of hydrocarbon-degrading bacteria section.

1.4 Applications of hydrocarbon-degrading bacteria

1.4.1 Bioremediation of oil contaminated sites

1.4.1.1 General introduction

There are both physical and chemical methods for the clean-up of oil contaminated sites, which are fairly well established. Physical methods include the use of booms, skimmers and sorbents, whilst chemical clean-up techniques involve the application of dispersants which accelerates the break up of oil into small droplets that sink below the surface and are gradually degraded by microorganisms (Prince, 1993). Other bioremediation techniques include: composting, which is an aerobic, thermophilic treatment that mixes contaminated material with a bulking agent; bioreactors, whereby biodegradation takes place in a container; bioventing, which is a method of drawing oxygen through the soil to stimulate microbial activity; biofilters treat air

emissions through the use of microbial stripping columns; bioaugmentation, which is the addition of bacterial cultures to contaminated medium and biostimulation, which stimulates the indigenous microbial population in soils or ground water by providing necessary nutrients (Boopathy, 2000). Bioremediation had been defined as “the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes” (Swannell *et al*, 1996). It is well known that the changes in the composition of petroleum and some of its processing products are mostly under the influence of microorganisms (Antic *et al*, 2006). Overall bioremediation is an attractive process due to its cost effectiveness and the benefit of pollutant mineralisation to carbon dioxide and water (Trindade *et al*, 2005).

1.4.1.2 Factors affecting bioremediation

Nutrient amendment (biostimulation) is a widely accepted technique for bioremediation; however, there is little understanding of the systematic effects of nutrient application on the degradative microbial population and the process of bioremediation (Head and Swannell, 1993). Although there is evidence that bioremediation is effective in the clean-up of oil contaminated sites, an important limitation is the inability to precisely formulate strategies that will result in predictable degradation rates and residual contaminant concentration. Nutrient concentration and hydrocarbon bioavailability are key factors affecting biodegradation rates of oil in contaminated sites (Xu *et al*, 2005). For example, the amount of nutrients (principally N and P) applied to spilled oil may be based on consideration of the amount of N and P required to convert a given amount of hydrocarbon to carbon dioxide, water and microbial biomass under oxic conditions or from the concentration of nutrients shown to support maximal growth rates of alkane-degraders in culture (Venosa *et al*, 1996). This empirical approach to bioremediation is mainly due to our limited understanding of the complexity of the biodegradative systems and microbial populations.

Resource-ratio theory is one of the major logical frameworks used in ecology to predict how competition for growth-limiting resources influences biological diversity and function within a biological community (Smith *et al*, 1998). When the quantitative requirements for a limiting resource and the growth and death rate of different

competing organisms are known, resource-ratio theory offers the possibility to predict the outcome of such interactions and therefore the required additions of resource (Head and Swannell, 1993). However, defining these parameters *in situ* is likely to be a difficult task and it may be some time before resource-ratio theory has an impact on operational bioremediation. Furthermore, on oiled beaches, microbial biomass is lost due to predation and physical removal by wave and tidal action; such factors must be considered in addition to the death rate of cells when extrapolating resource-ratio theory to field conditions (Venosa *et al*, 1996).

There are other fundamental practical issues to consider. It is important to determine the nutrient limitation status of the indigenous microbial population in order to identify if nutrients must be added to stimilate biodegradation. Nutrient measurements taken using chemical or instrumental methods may not reflect the bioavailability of nutrients, however analysis of genes expressed during nutrient starvation would allow accurate assessment of the nutrient limitation status (Fleming *et al*, 1993). In addition, there are factors such as pH, supply of oxygen, temperature, water availability and gas diffusion, which affect bioremediation and therefore need to be taken into consideration when formulating a bioremediation strategy for hydrocarbon contaminated sites (Bosecker *et al*, 1989).

The biodegradation of an oil-contaminated soil can also be seriously affected by the hydrocarbon structure, the contamination time and weathering processes, all of which alter the bioavailability of pollutants to microorganisms (Trindade *et al*, 2005). Weathering refers to the result of biological, chemical and physical processes that affect the type of hydrocarbons that remain in a soil. These processes enhance the sorption of hydrophobic organic contaminants to the soil matrix, decreasing the rate and extent of biodegradation (Trindade *et al*, 2005).

1.4.2 Microbial enhanced oil recovery

1.4.2.1 Heavy oil

At temperatures up to approximately 80˚C, petroleum in subsurface reservoirs is often biologically degraded over geological timescales by microorganisms that destroy hydrocarbons and other components to produce altered, denser 'heavy oils' (Head *et*

al, 2003). The effects of biodegradation of the composition and physical properties of crude oil and natural gases are well known. Oxidation of oil during biodegradation leads to a decrease in saturated hydrocarbon content (and to a smaller decrease in aromatic hydrocarbon content) and API gravity, a measure that correlates with economic value, whereas oil density, sulphur content, acidity, viscosity and metal content increase, which negatively affects oil productions (by reducing well-flow rates) and refining operations, reduces oil value (Roling *et al*, 2003).

1.4.2.2 Microbial enhanced oil recovery

Microbial technologies are commonly exploited in oil reservoirs to improve recovery. Injected nutrients, together with addition of indigenous microbes, promote *in situ* microbial growth and/or generation of products which mobilize recalcitrant oil deposits and move it to producing wells through reservoir repressurization, interfacial tension/oil viscosity reduction and selective plugging of the most permeable zones (Van Hamme *et al*, 2003). There are several factors which require consideration when applying this technology, such as the physiochemical properties of the reservoir in terms of salinity, pH, temperature, pressure and nutrient availability. In addition, only bacteria are considered promising candidates for microbial enhanced oil recovery because moulds, yeasts, algae and protozoa are not suitable due to their size or inability to grow under the conditions present in reservoirs (Van Hamme *et al*, 2003). A great deal of uncertainty surrounding microbial enhanced oil recovery exists despite much empirical evidence for its success. Microbial enhanced oil recovery requires an ability to manipulate environmental conditions to promote growth and/or product formation by the participating microorganisms. Exerting such control over the microbial system in the subsurface is a serious challenge. Furthermore conditions vary between reservoirs, which necessitate reservoir-specific customization of the microbial enhanced oil recovery process, and this alone has the potential to undermine microbial process economic viability (Van Hamme *et al*, 2003).

1.4.3. Bioaugmentation

Bioaugmentation for bioremediation is the utilisation of catabolically-relevant organisms (whether naturally occurring or genetically engineered variants) in order to hasten remediation of a contaminated site. Bioaugmentation can also be applied to

enhance oil recovery. Successful application of bioaugmentation techniques is dependent on the identification and isolation of appropriate microbial strains, and their subsequent survival and activity, once released into the target habitat (Boopathy, 2000). Sourcing microbial strains for bioaugmentation has typically been achieved by selective enrichment. This involves strains from polluted samples being enriched to grow, relative to the background community, in culture, using the target contaminant as the sole enriching carbon or nitrogen source. The procedure results in the selection of strains that express the required degradation ability in the specific conditions of the enrichment culture, however, the population may not possess other survival traits required *in situ* (Thompson *et al*, 2005). Trindade *et al* (2005) determined that soils submitted to bioaugmentation (utilizing a microbial consortium containing two crude oil degrading microorganisms) and biostimulation (adjusting C:N:P ratios) techniques presented biodegradation efficiencies approximately twice as high as the ones without the aforementioned treatments (natural attenuation). It is evident that this technique has potential, however, biodegradation can be inhibited by a multitude of factors, such as pH and redox, the presence of toxic co-contaminates, concentration, or the absence of key co-substrates (Thompson *et al*, 2005).

1.5 Polycyclic aromatic hydrocarbons (PAHs)

1.5.1 Environmental concerns about polycyclic aromatic hydrocarbons (PAHs)

Interest in the biodegradation mechanisms and environmental fate of PAHs is motivated by their ubiquitous distribution, their low bioavailability and high persistence in soil, and their potentially deleterious effect on human health (Ni Chadhain *et al*, 2006). PAHs are generated continuously by the inadvertent incomplete combustion of organic matter, for instance in forest fires, home heating, traffic, and waste incineration. Massive soil contamination with PAHs originated from extensive industrial coal gasification during most of the 20th century. As gas works were typically located in densely populated urban regions to facilitate the distribution of the coal gas, PAH contaminated sites are mostly found in or near cities, thus representing a considerable public health hazard (Johnsen *et* al, 2005). PAHs are also formed naturally in the environment, during such processes as thermal geological

reactions and natural fires; however, human activities are more significant contributors to the environment (DEFRA Air Quality report). On the basis of their abundance and toxicity, 16 PAH compounds have been identified by the U.S. Environmental Protection Agency (EPA) as priority pollutants, including naphthalene, anthracene and phenanthrene (Habe and Omori, 2003).

Boffetta *et* al (1997) reviewed the epidemiologic evidence on the relationship between PAHs and cancer. High occupational exposure to PAHs occurs in several industries and occupations such as: aluminium production, coal gasification, coke production, iron and steel foundries, tar distillation, shale oil extraction, wood impregnation, roofing, road paving, carbon black production, carbon electrode production, chimney sweeping, and calcium carbide production. In addition, workers exposed to diesel engine exhaust in the transport industry and in related occupations are exposed to PAHs and nitro-PAHs. Heavy exposure to PAHs engenders a substantial risk of lung, skin, and bladder cancer, which is not likely to be due to other carcinogenic exposures present in the same industries. The lung seems to be the major target organ of PAH carcinogenicity and increased risk is present in most of the industries and occupations listed above. An increased risk of skin cancer follows high dermal exposure. An increase in bladder cancer risk is found mainly in industries with high exposure to PAHs from coal tars and pitches. Increased risks have been reported for other organs, namely the larynx and the kidney. The available evidence, however, is inconclusive (Boffetta *et* al 1997).

PAHs are pervasive contaminants in rivers, lakes and nearshore marine habitats. Studies after the *Exxon Valdez* oil spill demonstrated that fish embryos exposed to low levels of PAHs in weathered crude oil develop a syndrome of edema and craniofacial and body axis defects. Although mechanisms leading to these defects are poorly understood, it is widely held that PAH toxicity is linked to aryl hydrocarbon receptor (AhR) binding and cytochrome P450 1A (CYP1A) induction. These findings have multiple implications for the assessment of PAH impacts on coastal habitats (Incardona *et al*, 2005).

1.5.2 Chemistry of PAHs

PAHs are composed of fused, aromatic rings whose biochemical persistence arises from dense clouds of π-electrons on both sides of the ring structures, making them resistant to nucleophilic attack (Johnsen *et al*, 2005). Different PAHs vary both in their chemical characteristics and in their environmental sources and they are found in the environment both as gases and associated with particles (DEFRA Air Quality report).

The simplest PAH is benzocyclobutene (C_8H_6) (Cerniglia, 1992). PAHs containing up to three benzene rings are known as low molecular weight PAHs and those containing more than four benzene rings are know as high-molecular-weight PAHs (Van Hamme *et al*, 2003). High-molecular-weight PAHs are more stable and are of higher toxicity than low-molecular-weight PAHs (Cerniglia, 1992). High-molecular-weight PAHs have low solubility in water and a low vapour pressure, as molecular weight increases, solubility and vapour pressure decrease. PAHs with two rings are more soluble in water and more volatile; because of this, PAHs in the environment are primarily associated with soil and sediment as opposed to water and air, however, PAHs are often found in particles suspended in water and air (Culotta *et al*, 2006). As molecular weight increases, the carcinogenicity of PAHs also increases and acute toxicity decreases.

Naphthalene ($C_{10}H_8$) is a fused ring bicyclic aromatic hydrocarbon and due to its simplicity serves as a model for understanding the properties of this large class of environmentally prevalent pollutants (Zeng, 2005). Naphthalene is a crystalline, aromatic, white, solid hydrocarbon and is volatile, forming a flammable vapour. Phenanthrene ($C_{14}H_{10}$) is a tricyclic aromatic hydrocarbon and has also been used as a model compound in PAH biodegradation studies (Shiaris *et al*, 1989; Lloyd-Jones *et al*, 1999; Moody *et al*, 2001; Cavalca *et al*, 2006). Anthracene is another tricyclic aromatic hydrocarbon, however, the positioning of the benzene rings differs to that seen in phenanthrene.

1.5.3 Microbial degradation of PAHs

A vast array of microbes (bacteria, fungi and algae) can utilise both low- and high-molecular-weight PAHs such as naphthalene, acenaphthalene, anthracene, fluoranthrene and pyrene as sole carbon and energy sources (Van Hamme *et al*, 2003). The most commonly isolated species with PAH-degradative capabilities are pseudomonads, with *Pseudomonas putida* the most frequently studied (Ahn *et al*, 1999; Cerniglia *et al,* 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 *et al*, 1992; Hamann *et al*, 1999).

Metabolism of PAHs involves the oxidation of the molecule via a multi-component enzyme system to form a dihydrodiol, which is then processed by either an *ortho* or *meta* cleavage pathway, which results in an intermediate. This is then further processed to form tricarboxylic acid cycle intermediates (Johnsen *et al*, 2005).

The genetic determination of naphthalene degradation has been greatly studied, with much focus on the naphthalene catabolic genes (*nah* genes) cloned from the NAH7 plasmid of *P. putida* strain G7 (*Pp*G7) (Zocca *et al*, 2004).

In the first catabolic step, an oxygen molecule is introduced at the 1,2-position of the aromatic nucleus to produce *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydroxynaphthalene (*cis*-naphthalene dihydrodiol) by naphthalene dioxygenase (NDO) (Habe and Omori, 2003). NDO is a multicomponent enzyme system, which consists of three components, a ferredoxin reductase, a ferredoxin and an iron-sulfur protein (ISP) composed of two non-identical subunits, α and β (Simon *et al*, 1993). The genes for the individual components of NDO in *P. putida* strains G7 (*Pp*G7) and NCIB 9816-4 (*Pp*9816-4) are encoded in the NAH7 plasmid and have been designated *nahAa* ($reductase_{NAP}$), *nahAb* ($ferredoxin_{NAP}$) and *nahAc* and *nahAd* (ISP_{NAP}, large and small subunits, respectively; Simon *et al*, 1993).

cis-Naphthalene dihydrodiol is then dehydrogenated to 1,2-dihydroxynapthalene by *cis*-naphthalene dihydrodiol dehydrogenase. 1,2-Dihydroxynaphthalene is *meta*-cleaved by 1,2-dihydroxynaphthalene dioxygenase, and the resulting ring-cleavage

product spontaneously recyclizes to form 2-hydroxy-2*H*-chromene-2-carboxylase. Enzymatic reactions by an isomerase and a hydratase-aldolase result in the production of salicylaldehyde, which is then transformed to salicylate by salicylaldehyde dehydrogenase. Salicylate is furthered metabolised *via* catechol or gentisate to TCA cycle intermediates (Habe and Omori, 2003).

Phenanthrene is degraded by some soil bacteria through one of two routes. In one route, 1-hydroxy-2-naphthoic acid is oxidized to 1,2-dihydroxynaphthalene, which is further degraded via the naphthalene pathway to salicylate, which can be furthered metabolised (Ouyang, 2005). In the other pathway, the ring of 1-hydroxy-2-naphthoic acid is cleaved by 1-hydroxy-2-naphthoate dioxygenase, later enzymatic reactions result in the production of phthalate, which is further metabolised *via* protocatechuate to TCA intermediates (Habe and Omori, 2003; Ouyang, 2005).

Anthracene is converted to 1,2-dihydroxyanthracene by an initial dioxygenolytic attack at the 1,2-position. A *meta*-cleavage pathway of 1,2-dihydroxyanthracene results in the production of 2-hydroxy-3-naphthoate, this is converted to 2,3-dihydroxynaphthalene, which is further metabolised to salicylate and catechol (Habe and Omori, 2003). The enzymes involved in the conversion of naphthalene to salicylate can degrade phenanthrene and anthracene to 1-hydroxy-2-naphthoate and 2-hydroxy-3-naphthoate, respectively, through similar catabolic steps (Habe and Omori, 2003); this demonstrates the broad substrate-specificity of the PAH-degradative genes.

1.5.4 Genetics of PAH degradation

Many PAH degrading pseudomonads possess genes involved in the PAH-biodegradative pathways that are highly homologous to the cluster of naphthalene genes (*nah* genes) cloned from the NAH7 plasmid of *Pp*G7 (Zocca *et al*, 2004). Sanseverino *et al* (1993) demonstrated the homology between PAH degradative genes from three *Pseudomonas* strains, designated 5R, DFC49 and DFC50. Plasmids pKA1, pKA2 and pKA3, approximately 100 kb in size, were isolated from these strains and characterised. These plasmids have homologous regions of upper and lower NAH7 plasmid catabolic genes and have been shown to encode the genotype for

mineralization of naphthalene, phenanthrene and anthracene (Sanseverino *et al*, 1993). The naphthalene catabolic plasmids (called NAH plasmids), such as pWW60 derivatives from *P. putida* strain NCIB 9816, pDTG1 from *P. putida* NCIB 9816-4 and pKA1 from *P. fluorescens* strain 5R, also were analyzed and the plasmids were found to be very similar to the NAH7 plasmid from strain G7 (Habe and Omori, 2003). Takizawa *et al* (1994) identified and characterised the gene cluster (*pah* genes) encoding the degradation of naphthalene and phenanthrene in *P. putida* OUS82. The *pahA* and *pahB* genes, which encode the first and second enzymes, dioxygenase and *cis*-dihydriol dehydrogenase, respectively, were identified and sequenced. The DNA sequence showed that *pahA* and *pahB* were clustered and that *pahA* consisted of four cistrons, *pahAa, pahAb, pahAc, pahAd*, which encode ferredoxin reductase, ferredoxin and two subunits of the iron-sulfur protein, respectively. The genomic structure and encoded enzymes are homologous to that seen in *Pp*G7 controlling naphthalene degradation and in fact the *pah* region of *P. putida* OUS82 strongly hybridised to a corresponding region of plasmid NAH7 of *Pp*G7 (Takizawa *et al*, 1994).

Nucleotide sequences of genes encoding the naphthalene upper-catabolic enzymes from several *Pseudomonas* strains were reported: *ndo* genes from *P. putida* strain NCIB 9816, *nah* genes from *P. putida* strain G7 and NCIB 9816-4, *dox* genes from *Pseudomonas* sp. strain c18, *pah* genes from *P. putida* strain OUS82 and *P. aeruginosa* strain PaK1, *nah* genes from *P. putida* strain BS202 (accession number AF010471) and *nah* genes from *P. stutzeri* strain AN10. The gene organisation and sequence similarity (about 90%) among the upper catabolic pathway genes of these strains were similar to those of the *nah* genes from the NAH7 plasmid of strain G7 (Habe and Omori, 2003).

The naphthalene catabolic plasmids (NAH plasmids) not only mediate degradation of naphthalene, but also phenanthrene, anthracene, dibenzothiophene, fluorene and methylated naphthalenes (Ahn *et al*, 1999) and the genes within these plasmids are therefore referred to as *nah*-like genes (Lloyd-Jones *et al,* 1999). Classical *nah*-like genes have almost identical organisation or nucleotide sequence in the Pseudomonads, which can be attributed to their presence in the NAH plasmids, which are in incompatibility group P7 or P9, are quite large, and are self-transmissible (Habe

and Omori, 2003). However, the nucleotide sequence homology and the organisation of isofunctional clusters of genes cloned from genera *Burkholderia, Comamonas, Ralstonia* and *Sphingomonas* are not so conserved in respect with the *nah* genes (Zocca *et al*, 2004).

The naphthalene catabolic genes within the well-studied *Pp*G7 strain are organised into three operons on the 83-kb NAH7 plasmid: one encoding the upper-pathway enzymes involved in the conversion of naphthalene to salicylate, the second encoding the lower pathway enzymes responsible for converting salicylate into the TCA cycle intermediate and the third encoding the regulatory protein NahR (Habe and Omori, 2003). Both the upper and the lower operons are regulated by a *trans*-acting positive control regulator encoded by the *nahR* gene. NahR is needed for the high-level expression of the *nah* genes and their induction by salicylate (Park *et al*, 2002).

Comamonas testosteroni strains GZ38A, GZ39 and GZ42 are capable of degrading phenanthrene, naphthalene and anthracene, however, there was no homology between the degradative genes within these strains and the classical *nah*-like genes from *Pp*9816-4 (Goyal and Zylstra, 1996). Molecular analysis of the first two genes for phenanthrene degradation, phenanthrene dioxygenase and *cis*-phenanthrene dihydrodiol dehydrogenase, revealed a novel organisation of these genes. It was demonstrated that *C. testosteroni* GZ38A possessed phenanthrene-degradative genes similar to those cloned from *C. testosteroni* GZ39. However, *C. testosteroni* GZ42 possessed genes mediating phenanthrene degradation that were not homologous to those from *C. testosteroni* GZ39, which suggests there are at least two different sets of catabolic genes determining phenanthrene degradation in these *C. testosteroni* strains (Goyal and Zylstra, 1996).

Burkholderia sp. strain RP007 was isolated from a PAH-contaminated site in New Zealand and is capable of degrading phenanthrene, naphthalene and anthracene (Laurie and Lloyd-Jones, 1999). Naphthalene and phenanthrene are degraded through a common pathway *via* salicylate and 1-hydroxy-2-naphthoic acid, respectively. *phn* genes [encoding regulatory proteins (*phnR* and *phnS*), aldehyde dehydrogenase (*phnF*), hydratase-aldolase (*phnE*), extradiol dehydrogenase (*phnC*), isomerase (*phnD*), ISP α subunit of initial dioxygenase (*phnAc*), ISP β subunit of initial

dioxygenase (*phnAd*), dihydrodiol dehydrogenase (*phnB*), in this order] were different in nucleotide sequence and gene organisation from previously characterised PAH-catabolic genes (Laurie and Lloyd-Jones, 1999). For example, the locus contains ISP α and β subunits of PAH-initial dioxygenase (*phnAcAd*) but lacks both the ferredoxin and reductase components (Habe and Omori, 2003).

It has been established that microbial species can utilize PAHs as growth substrates, with the mechanism for degradation and genetic control understood. The literature contains conflicting reports of the ability of different microbial species to degrade multiple PAHs. There is also conflict as to the homology of PAH-catabolic genes from different species. Since the initial step of the degradation of PAHs involves the introduction of an oxygen molecule, it seems likely that the gene controlling this step will encode an enzyme with a broad-enzymatic activity towards other PAHs. It is important to determine if there are primer sets which are capable of detecting PAH-degradative genes, capable of degrading multiple PAHs, within different species so as to identify all components of a diverse environmental catabolic gene pool.

Following the interest of the *nah* genes cloned from the NAH7 plasmid of *Pp*G7 (Zocca *et al*, 2004) other PAH catabolic genes have been sequenced (e.g. Laurie and Lloyd-Jones, 1999), however much of the research has focused on a particular gene sequence identified from a single strain. This research has focused on the gene encoding the initial dioxygenase as this step seems to be common to PAH degradation. It is important to investigate the evolutionary relationships between all identified PAH-dioxygenase genes, in order to understand the transmission within microbial populations. In addition, phylogenetic analysis of all reported PAH-dioxygenase genes will indicate the homology of these genes across all identified PAH-degrading bacteria.

1.5.5 Naphthalene dioxygenase structure and the role of functionally important amino acid residues

Naphthalene dioxygenase (NDO) is a member of a large family of bacterial Rieske non-heme iron oxygenases that initiate the degradation of a wide range of aromatic compounds in aerobic environments (Parales *et al*, 2003). These multicomponent

enzyme systems are capable of oxidizing aromatic hydrocarbons, aromatic acids, chlorinated aromatic compounds, as well as nitroaromatic and amino aromatic compounds (Parales *et al*, 2003).

The study of Rieske non-heme iron oxygenases is important for two reasons. First, these enzymes catalyze essential reactions in bacterial pathways for the degradation of many aromatic compounds that are considered to be serious environmental pollutants. Secondly, enzymes such as NDO have broad substrate ranges (NDO, for example, is known to oxidize over 60 different aromatic substrates). A particularly interesting feature of NDO is its ability to catalyze different types of reactions besides *cis*-dihydroxylation. In addition to dioxygenation, NDO has been shown to catalyze monooxygenation, desaturation, sulfoxidation and dealkylation reaction (Parales *et al*, 2003). NDO catalyses the first step in the aerobic degradation of naphthalene.

Parales *et al* (1999) have investigated the role of active-site residues in NDO. NDO catalyses the first step in the aerobic degradation of naphthalene. NDO adds two atoms of oxygen to the aromatic nucleus of naphthalene, forming *cis*-naphthalene dihydrodiol (Parales *et al*, 2000). The NDO system consists of three components. An iron-sulfur flavoprotein reductase and an iron-sulfur ferredoxin transfer electrons from NAD(P)H to the catalytic oxygenase component (figure 1.1; Parales *et al*, 2000). The reduced oxygenase then catalyzes the stereospecific addition of two atoms of molecular oxygen to the aromatic nucleus of the substrate.

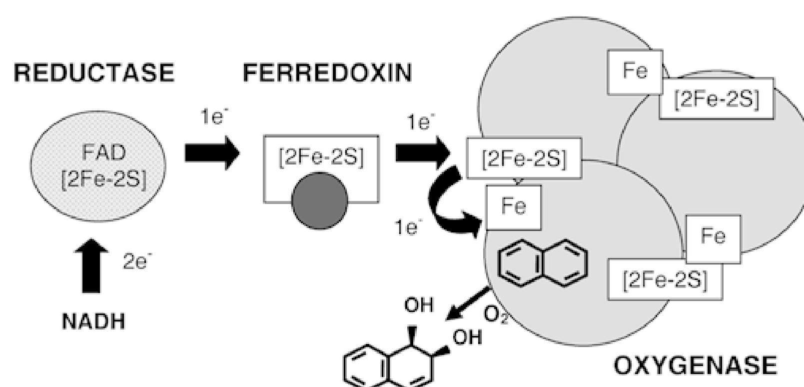

Figure 1.1: Reaction catalysed by the multicomponent naphthalene dioxygenase system (Parales *et al*, 1999).

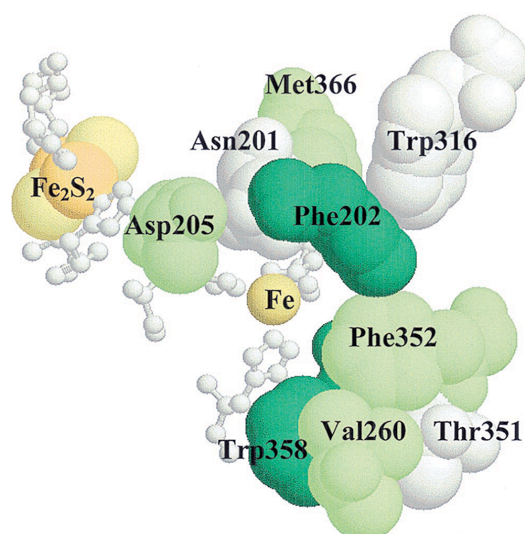

Figure 1.2: Structure of the active site of NDO, showing the Rieske [2Fe-2S] center and mononuclear iron in adjacent α subunits with their coordinating amino acids (shown in white ball-and-stick format). Also shown is Asp-205, an amino acid important for efficient electron transfer between the two redox centers (Parales *et al*, 2000).

With the completion of the three-dimensional crystal structure of naphthalene dioxygenase, the role of each of the amino acids in catalysis can be clarified. The crystal structure revealed that the oxygenase is composed of large and small subunits, α and β, respectively, that are arranged as an $\alpha_3\beta_3$ hexamer (Parales *et al*, 1999). Each α-subunit contains two domains, a Rieske [2Fe-2S] center and a mononuclear non-heme iron (Parales *et al*, 2000). In the NDO system, electrons are transferred from the Rieske center of the ferredoxin to the Rieske center of the oxygenase α subunit (figure 1.1; Parales *et al*, 2000). It has been proposed that the active site is located at the junction of two α subunits and that electrons are transferred from the Rieske center of one subunit to the mononuclear iron of the adjacent α subunit (Parales *et al*, 1999). Recent studies have shown that the oxygenase α subunits are responsible for determining the substrate specificities of NDO (Parales *et al*, 2000).

The crystal structure of naphthalene dioxygenase indicates that aspartate-205 (Asp-205) may provide the most direct route of electron transfer between the Rieske center of one α subunit and mononuclear iron in the adjacent α subunit (Parales *et al,* 1999). The Rieske [2Fe-2S] center is coordinated by Cys-81, Cys-101, His-83 and His-104. The one atom of mononuclear ferrous iron at the active site of the α subunit is

coordinated by His-208, His-213 and Asp-362. These seven ligand-binding residues are conserved in all Rieske non-heme iron oxygenases and a consensus has been derived (figure 1.3). Asp-205 in the catalytic domain of the NDO α subunit is hydrogen bonded to His-208 and His-104 in the adjacent α subunit (Parales *et al*, 2000). Amino acids near the active-site iron atom in the catalytic domain of the α subunit are Asn-201, Phe-202, Val-260, Trp-316, Phe-352, Trp-358 and Met-366, which are close enough to the mononuclear iron to interact with substrates in the active site (figure 1.2; Parales *et al*, 2000). Asn-201 is positioned too far from the iron atom to be a ligand in the crystallized form of NDO, but was suggested as a possible ligand during some stage of the catalytic cycle (Parales *et al*, 2000). Asp-362 is one of the three amino acids that coordinate the iron at the active site and replacement with alanine disrupted iron coordination (Parales *et al*, 2000).

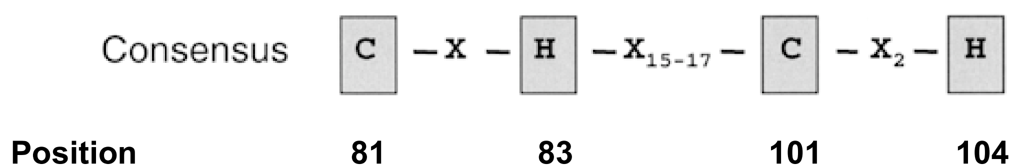

Figure 1.3: Alignment consensus of amino acid residues in the Rieske centre binding site (Parales *et al*, 2003). C represents cistine, H represents histidine and x can be any amino acid residue.

1.6 Polycyclic aromatic compounds (PACs)

1.6.1 Dibenzothiophene (DBT)

Sulphur is usually the third most abundant element in crude oil, normally accounting for 0.05-5%, but this value increases to 14% in heavier oils. Most of the sulphur in crude oil is organically bound, mainly in the form of condensed thiophenes (Van Hamme *et al*, 2003); DBT is a representative of this group of sulphur-containing heterocyclic organic compounds (Laborde *et al*, 1997). Dibenzothiophene (DBT) has been found to also exist in coal, airborne particulates, storm water runoff and in sediments and organisms (Yang *et al*, 1997). Interest in 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, relative to the other aromatic compounds found in crude oils has also been observed

in other studies and contributes to the potential of condensed thiophenes to accumulate in the tissues of shellfish in marine environments that become contaminated with crude oil (Kropp *et al*, 1997).

1.6.2 The genetic control of the degradation of DBT

The degradation of DBT and its associated genetic control has been studied. The degradation of DBT by *Pseudomonas* strain C18 is mediated by the DOX pathway and the nine open reading frames within the 9.8 kb DNA fragment encoding the DBT-degrading enzymes were identified and designated *doxABDEFGHIJ* (Denome *et al*, 1993). It has been elucidated that the DOX operon is very similar to that of the naphthalene pathway. Comparison of the DOX sequence with restriction maps of cloned naphthalene catabolic pathway (NAH) revealed many conserved restriction sites. At the nucleotide level, *doxABC* are identical to the *ndoABC* genes that encode naphthalene dioxygenase and DoxG protein is 97% identical to NahC of *Pseudomonas putida* (Denome *et al*, 1993).

Van Herwijnen *et al* (2003) have reported the elucidation of the metabolic pathway of fluorene and cometabolic pathways of phenanthrene, fluoranthrene, anthracene and DBT by *Sphingomonas* sp. LB126 (Van Herwijnen *et al*, 2003). This is the first study demonstrating the cometabolic degradation of the three-ring PAHs phenanthrene, anthracene and the four-ring PAH fluoranthrene by a fluorene-utilising species and affirms the broad specificity of the degradative systems within bacteria towards PACs.

1.7 Investigating the evolution of functional genes

Use of 16S rRNA and functional gene diversity analyses allows the inference of functional gene evolutionary history. This comparative molecular approach revealed multiple lateral transfers of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes (Klein *et al*, 2001). Additionally, comparative phylogenetic analysis revealed multiple lateral transfers of adenosine-5'-phophosulfate reductase genes among sulfate-reducing microorganisms (Friedrich, 2002).

Determining the congruency of the tree topologies for functional genes and the corresponding 16S rRNA from degradative populations can allow conclusions to be drawn of the evolution of the functional genes. If lateral spread of the functional genes has occurred between degradative species, one would expect the lineage of the transferred gene to be phylogenetically less diverged (i.e. more closely related) than that of the host cells (Herrick *et al*, 1997). This is based on the assumption, which is expected, that the mutation rate of the 16S rRNA gene is lower than that of the functional gene. Moreover, the phylogeny of the 16S rRNA and functional gene would not be expected to be congruent. Conversely, lack of horizontal gene transfer would be indicated if the lineage of the biodegradation gene were diverged to an extent equal to or more than the 16S rRNA (Herrick *et al*, 1997). The 16S rRNA gene was selected based on the following characteristics of the gene as outlined by Woese: (i) there is no evidence of horizontal transfer of the 16S rRNA gene of portions thereof; (ii) it is highly conserved, with significant sequence identity retained even across domain and kingdom boundaries; and (iii) its phylogeny is considered representative of the phylogeny of the organism possessing it (Herrick *et al*, 1997).

Understanding the extent, frequency and mechanisms of gene transfer between microorganisms may advance knowledge of the evolution of bacterial populations and also improve strategies for manipulating these populations to enhance microbial detoxification of pollutant compounds *in situ*.

1.8 The distribution and diversity of PAC-degrading bacteria and key degradative genes

ANT, PHE and DBT have been highlighted as having carcinogenic and mutagenic properties together with a near ubiquitous distribution in depositional environments (Yang *et al*, 1998). Polycyclic aromatics compounds (PACs) such as the above mentioned, pose great environmental concern and therefore require investigative research to development strategies to remove PACs from contaminated sites.

PAHs are composed of fused aromatic rings and they possess physical properties, such as low aqueous solubility and high solid-water distribution ratios, which makes PAHs less bioavailable for microbial utilisation and promote their accumulation in solid phases of the terrestrial environment (Johnsen *et al*, 2005). Previous research has

been conducted into the biodegradation of PAHs for the development of bioremediation strategies for PAH-contaminated sites. Frequently molecular ecological approached are combined with traditional laboratory enrichments to identify bacterial populations that are functionally important in biodegradation of organic pollutants (Daane *et al*, 2001; Launen *et al*, 2008; Mueller *et al*, 1997). In the laboratory, enrichment of bacteria able to utilise PAHs as the carbon source has mostly been performed in shaking liquid media, whereby PAHs are added dissolved in a carrier solvent (e.g. Izumi *et al*, 1994; Moody *et al*, 2001; Nadalig *et al*, 2002). Using such an experimental approach results in enrichment of a microbial population that may not represent a PAH-degradative community and may lead to inaccurate conclusions to be drawn. The literature does not seem to determine the specific effects that the use of a carrier solvent may have on microbial community structure; the effects of which may have significant implications when developing bioremediation strategies.

Understanding how bacteria acclimatise to environmental pollutants is vital for exploiting these mechanisms within clear up strategies of contaminated sites. There is evidence for the lateral transfer of PAH-degradative genes (Herrick *et al*, 1997; Wilson *et al*, 2003) however to broaden our understanding it is important that all previously reported analogs of a functionally important gene, such as naphthalene dioxygenase, be phylogenetically analysed so that conclusions may be drawn of the evolution of this gene. Determining the extent to which this gene can be laterally transferred between the divisions of bacteria will have important consequences when developing bioaugmentation techniques and may result in other practical implications e.g. primer and probe design.

1.9 Research objectives

A focus of this study is to determine the effect of using a carrier solvent when performing pollutant-degradation experiments, in order to optimize this experimental approach. Once optimization of this technique is achieved, it will be used to isolate and identify key polycyclic aromatic compounds-degrading bacteria. To further understand how bacteria within microbial populations adapt to the presence of pollutants e.g. polycyclic aromatic hydrocarbons, phylogenetic methods will be used to investigate the transfer of an important gene, the α subunit of PAH dioxygenases, between bacterial species.

- Determine the effect of a carrier solvent on the resultant microbial community structure when enriching for a hydrocarbon-degrading bacterial population and therefore assess the suitability of such a technique for extrapolation to *in situ* conditions during bioremediation.

- Isolate and identify key PAC-degrading bacteria, enriched from a sediment site, with the ability to degrade three significant environmental pollutants (ANT, PHE, DBT) that may be utilised in future bioremedation programmes of contaminated sediments.

- The use of phylogenetic methods to investigate the evolution of the α subunit of PAH dioxygenases, which will indicate the adaptability of microbial communities to degrade PAHs and other environmental pollutants. Knowledge of the extent and frequency of lateral transfer of the α subunit of PAH-dioxygenases will contribute to our ability to exploit bacterial PAH-degradative capabilities and enable the development of bioremediation strategies.

CHAPTER TWO

The impact of the use of a carrier solvent on the enrichment of hydrocarbon-degrading bacterial communities

2.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants and are generated from anthropogenic activities such as the burning of fossil fuels, the use of wood preservatives such as creosote and the generation of wastes from coal gasification plants (Ni Chadhain *et al,* 2006). Their widespread distribution and potentially deleterious effects on human health has been the impetus behind most of the interest in the biodegradation mechanisms and environmental fate of PAHs.

PAHs are composed of fused aromatic rings and they possess physical properties, such as low aqueous solubility and high solid-water distribution ratios, which makes PAHs less bioavailable for microbial utilisation and promote their accumulation in solid phases of the terrestrial environment (Johnsen *et al*, 2005). PAHs in the environment are primarily associated with soil and sediment as opposed to water and air, however, PAHs are often found in particles suspended in water and air (Culotta *et al*, 2006). Despite this, a vast array of microbial species (bacteria, fungi and algae) can utilise both low- (three rings or fewer) and high-molecular-weight (four or more rings) PAHs such as naphthalene, acenaphthalene, anthracene, fluoranthrene and pyrene as sole carbon and energy sources (Van Hamme *et al*, 2003).

Previous research has been conducted into the biodegradation of PAHs for the development of bioremediation strategies to decontaminate PAH-contaminated sites. Frequently, molecular ecological approaches are combined with traditional laboratory enrichments to identify bacterial populations that are functionally important in the biodegradation of organic pollutants (Daane *et al*, 2001; Launen *et al*, 2008; Mueller *et al*, 1997).

In the laboratory, enrichment of bacteria able to utilise PAHs as the carbon source has mostly been performed in shaking liquid media, whereby the addition of PAHs is dissolved in a carrier solvent (e.g. Izumi *et al*, 1994; Moody *et al*, 2001; Nadalig *et al*, 2002). This experimental approach affects the subsequent enriched microbial populations in two significant ways. Firstly, the carrier solvent provides a second carbon source and therefore the resultant population can not strictly be identified as PAH-degraders and secondly, PAH bioavailability is reduced or entirely diminished when PAHs interact with non-aqueous phase liquids (NAPL; Cavalca *et al*, 2008) and therefore the use of some solvents as carrier solvents may result in the PAHs being unavailable to the degrading bacteria. Microorganisms adopt several strategies to release xenobiotics at a faster rate: release of extracellular enzymes, regulation of uptake systems, production of biosurfactants, depletion of the xenobiotic concentration in the surrounding volume, change of cell surface hydrophobicity (Beal and Betts, 2000; Bouchez-Naitali *et al*, 1999; Cavalca *et al*, 2008; Pirog *et al*, 2004). The use of a carrier solvent would favour microorganisms that are able to adopt these strategies and therefore enrich for a subsection of the population.

An alternative experimental approach involves the use of PAH crystals within a batch liquid system (Ahn *et al,* 1999; Andreoni *et al*, 2004; Chadhain *et al*, 2006). Degradation of crystalline PAHs in suspended shaken cultures is believed to be limited by the amount dissolved in the water phase, however, microbial populations exhibit characteristic growth curves (Johnsen *et al*, 2005). Although the state in which the PAHs would be available in this experimental approach are different to that experienced by microbial populations *in situ*, this enrichment technique ensures that the resultant population is capable of utilising PAHs as a sole carbon and energy source rather than selecting for species that utilise the

carrier solvent. The use of PAH crystals allows the direct isolation and identification of PAH-degrading species.

This study determined the effect of the use of acetone as a carrier solvent on the rates of degradation of anthracene (ANT) and phenanthrene (PHE) and the bacterial species that comprised the resultant populations. Acetone has been used as a carrier solvent as it is miscible in aqueous solutions and therefore does not form a separate phase to the liquid media, it does, however, provide an alternative carbon source to the PAHs. The presence of acetone significantly reduced the rate of degradation of PAH substrates. Although the same sediment sample was used as the bacterial source in all enrichments, different bacterial strains were obtained depending upon the presence of the carrier solvent and the PAH. The species comprising the resultant population of the enrichment when a carrier solvent was present were similar to previously reported PAH-degrading species. This is significant when considering methodology for future isolation of PAH-degrading bacteria. In addition, such a finding necessitates review of previously reported PAH-degrading bacterial species that have been isolated and identified from enrichments using a carrier solvent.

2.2 Materials and Methods

Collection of environmental samples. Sediment samples from the Severn Estuary (Severn Bridge, UK) were collected for enrichment of PAH-degrading consortia in sterile universal tubes.

Enrichment cultures. Triplicate cultures were established in 50 ml flasks using 1 g Severn Bridge sediment and 25 ml minimal salts media (MSM; Tett *et al*, 1994) with the addition of the following as substrates to a final concentration of 200 mg/l: ANT crystals; PHN

crystals; ANT dissolved in acetone; PHE dissolved in acetone and acetone. The acetone enrichments contained 50 μl acetone, in order to contain equivalent volume of acetone as the enrichments that utilised a carrier solvent. 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. Absorbances of the cultures were measured at 420_{nm} against an abiotic MSM control to monitor bacterial growth.

PAH analysis. At each time point the enrichment cultures were centrifuged at 9710 × g for 10 mins. The supernatant was 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 6800 × 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 pH8.0), then 0.5 ml phenol:chloroform:isoamyl alcohol (pH8.0) (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 seconds to lyse the cells. The tubes were then centrifuged at 11337 × g for 5 minutes. 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 11337 × 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 Units/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 minutes × 1 cycle; 95°C 1 minute, 55°C 1 minute, 72°C 2 minutes × 30 cycles; 72°C 5 minutes × 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 AutoChemiSystem (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, PHN and DBT. PCR amplification was performed using eubacterial primers (PA and PH). PA forward primer: 5'-AGAGTTTGATCCTGGCTCAG-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.

2.3 Results

Bacterial growth and PAC degradation

Enrichment cultures were monitored over time to examine the growth and PAH degradation when enriched on: ANT crystals; ANT using acetone as a carrier solvent; PHE crystals; PHE using acetone as a carrier solvent; acetone. Optical density measurements (420_{nm}) of the enrichments show an increase in biomass over time (figure 2.1, 2.2, 2.3, 2.4). The ANT crystals enrichment culture showed no lag phase before the onset of exponential growth and reached a maximum optical density of 0.73 (figure 2.1) whilst the enrichment culture on ANT using a carrier solvent resulted in a maximum optical density of 0.96 after

37 days enrichment (figure 2.2). The PHE crystal 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 (figure 2.3), whereas the enrichment culture established on PHE using a carrier solvent reached an optical density of 1.0 after 37 days enrichment period (figure 2.4).

ANT degradation by the enrichment culture showed a decrease from 200 mg/l to approximately 50 mg/l by day 5 (figure 2.1) whereas ANT degradation by the enrichment established on ANT dissolved in acetone only showed a decrease from 200 mg/l to 190 mg/l (figure 2.2). PHE degradation by the enrichment culture showed a decrease from 200 mg/l to 75 mg/l by day 4 (figure 2.3) while PHE degradation by the enrichment established on PHE dissolved in acetone only showed a decrease from 200 mg/l to 184 mg/l (figure 2.4).

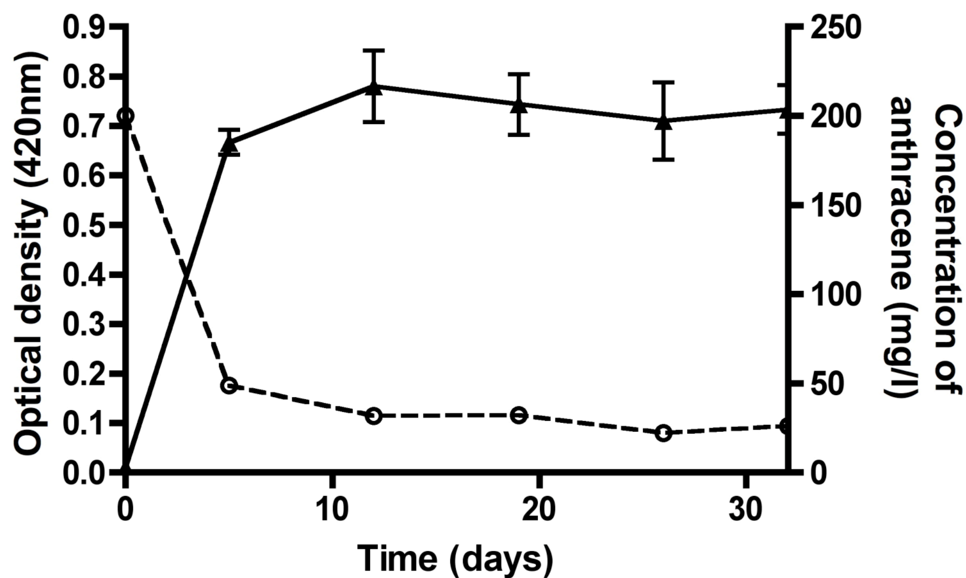

Fig. 2.1 Microbial growth (▲) and biodegradation of ANT (**O**) over time. Culture enriched on ANT crystals. Data represents the averages and standard errors of triplicate data.

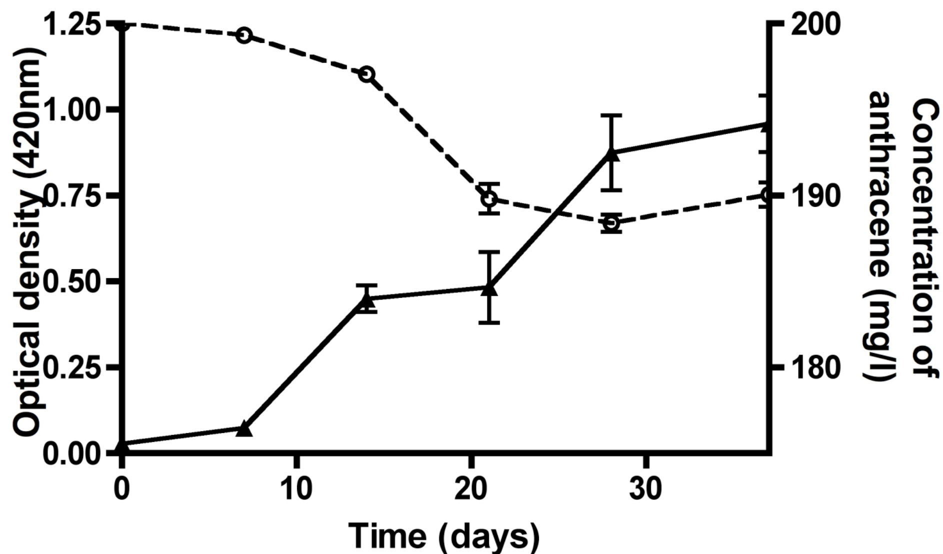

Fig. 2.2 Microbial growth (▲) and biodegradation of ANT (O) over time. Culture enriched on ANT with acetone present. Data represents the averages and standard errors of triplicate data.

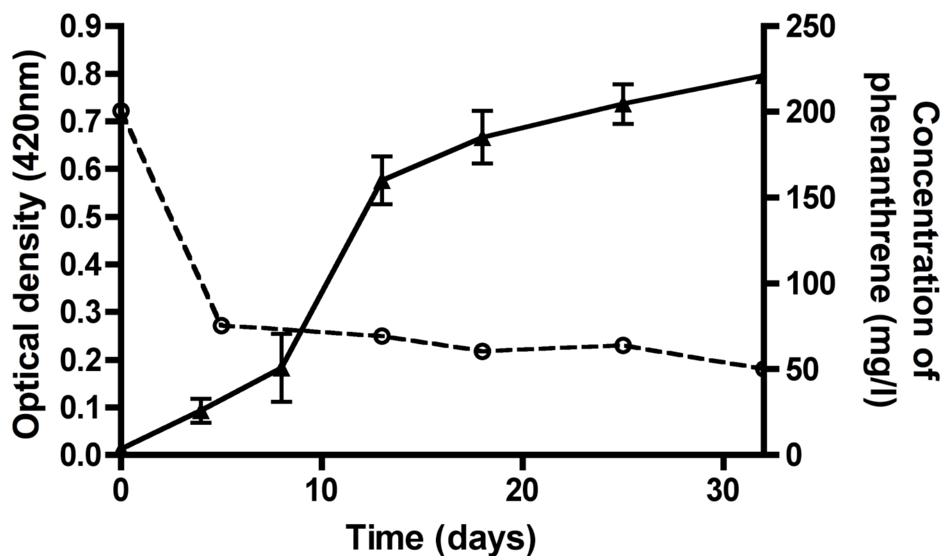

Fig. 2.3 Microbial growth (▲) and biodegradation of PHE (O) over time. Culture enriched on PHE crystals. Data represents the averages and standard errors of triplicate data.

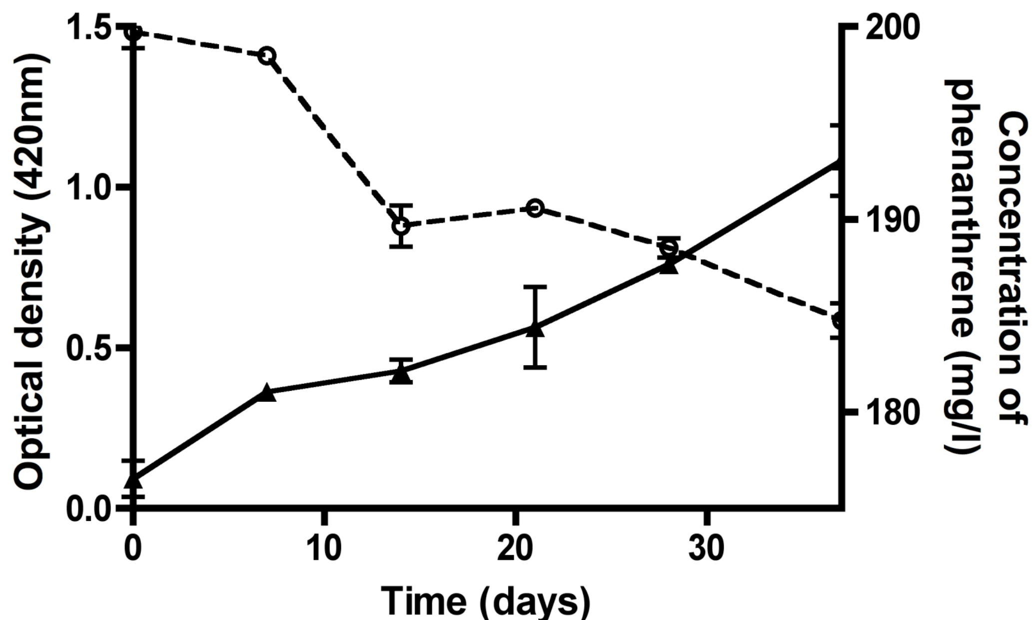

Fig. 2.4 Microbial growth (▲) and biodegradation of PHE (O) over time. Culture enriched on PHE with acetone present. Data represents the averages and standard errors of triplicate data.

Microbial growth was monitored within the culture enriched with acetone only (figure 2.5). The acetone enrichment culture showed an increase in optical density to 0.1 after 37 days enrichment. The final optical density measurement of the acetone enrichment was approximately 11% of that in the ANT enrichment (with acetone as carrier solvent) and approximately 10% of that in the PHE enrichment (with acetone as carrier solvent).

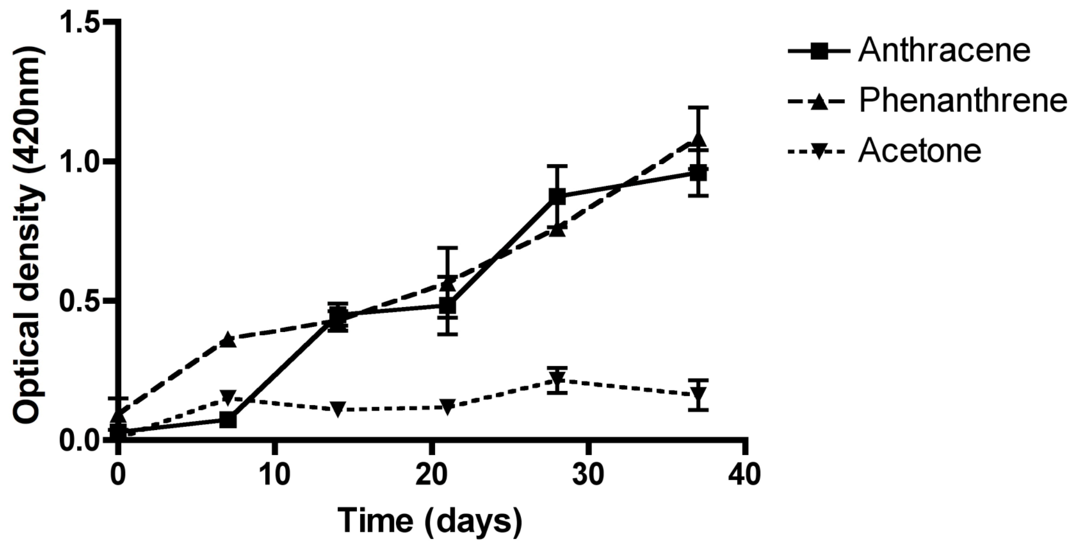

Fig. 2.5 Microbial growth of the culture when enriched on anthracene with acetone present (■), phenanthrene with acetone present (▲) and acetone only (▼). Data represents the averages and standard errors of triplicate data.

Bacterial community structure

The effect of enrichment with ANT/PHE crystals and ANT/PHE in the presence of a carrier solvent on the bacterial community structure was determined throughout the time course using 16S rRNA gene-based PCR-DGGE for each treatment (figure 2.6). 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 all the enrichments were observed by the second time point (8, 4, 8, 4 days respectively). DGGE analysis of cultures in each enrichment resulted in unique banding profiles, implying the selection of distinct substrate-specific bacterial populations.

The DGGE profiles generated from the PAH enrichments with and without a carrier solvent (figure 2.6) are different. When acetone is present the number of prominent bands at the final time point is fewer than when there is no carrier solvent present. PHE enrichment using acetone resulted in approximately 3 prominent bands by day 37, whilst the enrichment without acetone resulted in approximately 13 prominent bands. Anthracene

enrichment using the carrier solvent resulted in approximately 7 prominent bands by day 37, whilst the enrichment without the carrier solvent resulted in approximately 11 prominent bands. The position of the bands within the enrichments using acetone is at a higher denaturant concentration than the bands within the enrichments without acetone. These profiles imply distinct microbial populations are established when acetone is used as a carrier solvent.

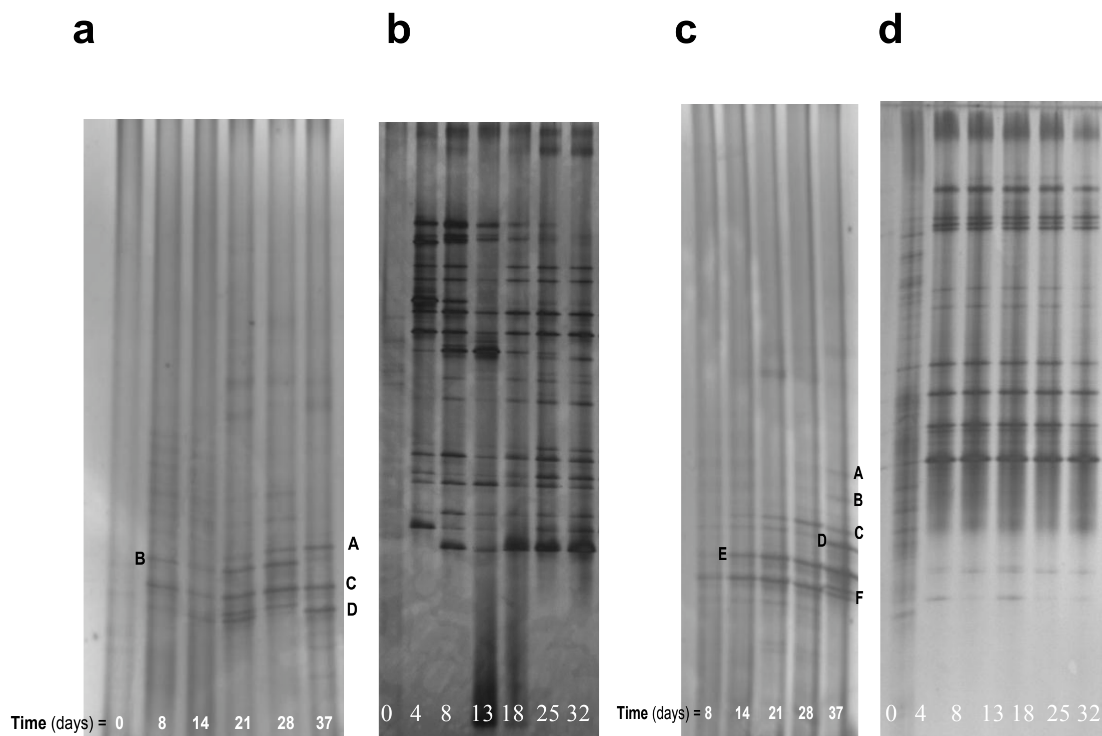

Fig. 2.6 DGGE fingerprints of the 16S rRNA genes in the Severn Bridge culture when enriched with phenanthrene dissolved in acetone (a), phenanthrene without the carrier solvent (b), anthracene dissolved in acetone (c) and anthracene without the carrier solvent (d). The highlighted bands have increased in intensity over the enrichment period and therefore may represent key bacterial species within the enrichments.

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 crystals, ANT/PHE with a carrier solvent present and acetone. A total of 250 clones were obtained from the enrichments. The clone libraries were screened using DGGE analysis and 36 different clones were selected

for complete 16S rRNA sequence analysis. The sequence identity of the selected clones revealed that the ANT enrichment with a carrier solvent present contained three clones identified as *Pseudomonas* spp., two as *Rhodococcus* spp., one as *Achromobacter xylosoxidans* and one as *Alcaligenes faecalis* (table 2.1). The ANT crystal 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 2.1).

Carrier solvent			**No carrier solvent**		
Closest genera	**Accession no.**	**% sequence homology**	**Closest genera**	**Accession no.**	**% sequence homology**
Rhodococcus erythropolis	EF052854	*99*	*Xanthobacter* sp. MN 45.1	AJ313028	99
Pseudomonas marginalis	DQ232743	97	Uncultured *Bradyrhizobium* sp.	DQ917252	97
Pseudomonas fluorescens	DQ178234	99	*Rhizobium daejeonense* L22	DQ089696	96
Rhodococcus sp.	DQ090961	100	*Xanthomonas oryzae*	AP008229	97
Achromobacter xylosoxidans	DQ659433	99	Bacterium RBS4-92	AJ536689	99
Alcaligenes faecalis	AJ509012	99	*Bradyrhizobium* sp.	AY238503	99
Pseudomonas sp.	AF195777	99	*Mycoplana peli*	EU256383	96
			Pseudomonas boreopolis	AB246809	97

Table 2.1. Closest genera and percentage sequence homology of the 16S rRNA clone library isolated from the ANT enrichments.

The PHE enrichment with a carrier solvent present contained three *Rhodococcus* spp., two *Pseudomonas* spp., two *Bosea* spp., one *Stenotrophomonas maltophilia* and one *Xanthobacter* sp. (table 2.2). The PHE crystal enrichment contained one clone with closest sequence identity to *Nocardia* sp., one clone as *Alcaligenes* sp., one clone as *Achromobacter xylosoxidans* and one clone as *Pseudomonas* sp. (Table 2.2).

Carrier solvent			**No carrier solvent**		
Closest genera	**Accession no.**	**% sequence homology**	**Closest genera**	**Accession no.**	**% sequence homology**
Rhodococcus erythropolis	AY833097	99	Uncultured soil bacterium	DQ297971	97
Bosea minatitlanesis	AF273081	97	*Norcardiaceae* bacterium	EF028121	99
Stenotrophomonas maltophilia	AY512625	99	*Alcaligenes faecalis*	AJ509012	99
Pseudomonas sp.	DQ219370	99	*Achromobacter xylosoxidans*	DQ659433	99
Bosea sp.	AJ313022	99	*Pseudomonas boreopolis*	AJ864722	97
Rhodococcus erythropolis	DQ397663	98			
Pseudomonas sp.	AF195777	99			
Rhodococcus sp.	DQ090961	100			
Xanthobacter sp.	AJ313028	99			

Table 2.2 Closest genera and percentage sequence homology of the 16S rRNA clone library isolated from the PHE enrichments.

The acetone enrichment contained two *Achromobacter xylosoxidans* strains, two β-*Proteobacteria*, one *Bordetella* sp. and one *Hydrogenophaga* sp. (table 2.3)

Closest genera	**Accession no.**	**% sequence homology**
Bordetella bronchiseptica	BX640447	100
Uncultured *beta Proteobacterium*	AY695723	99
Achromobacter xylosoxidans	DQ466568	99
Hydrogenophaga sp.	DQ986320	98
Achromobacter xylosoxidans	AF467978	99
Beta Proteobacterium	AY162060	98
Unidentified bacterium	AY345517	99

Table 2.3. Closest genera and percentage sequence homology of the 16S rRNA clone library isolated from the culture enriched on acetone only.

Phylogenetic analysis was performed on the degradative communities isolate from the ANT/PHE crystal and acetone enrichments.

All 16S rRNA sequences were aligned and neighbour-joining trees were constructed (Figure 2.7). 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). All the 16S rRNA gene clones from acetone enrichment were in the β-*Proteobacteria* (acetone 3, 4, 12, 24, 27, 36, 40).

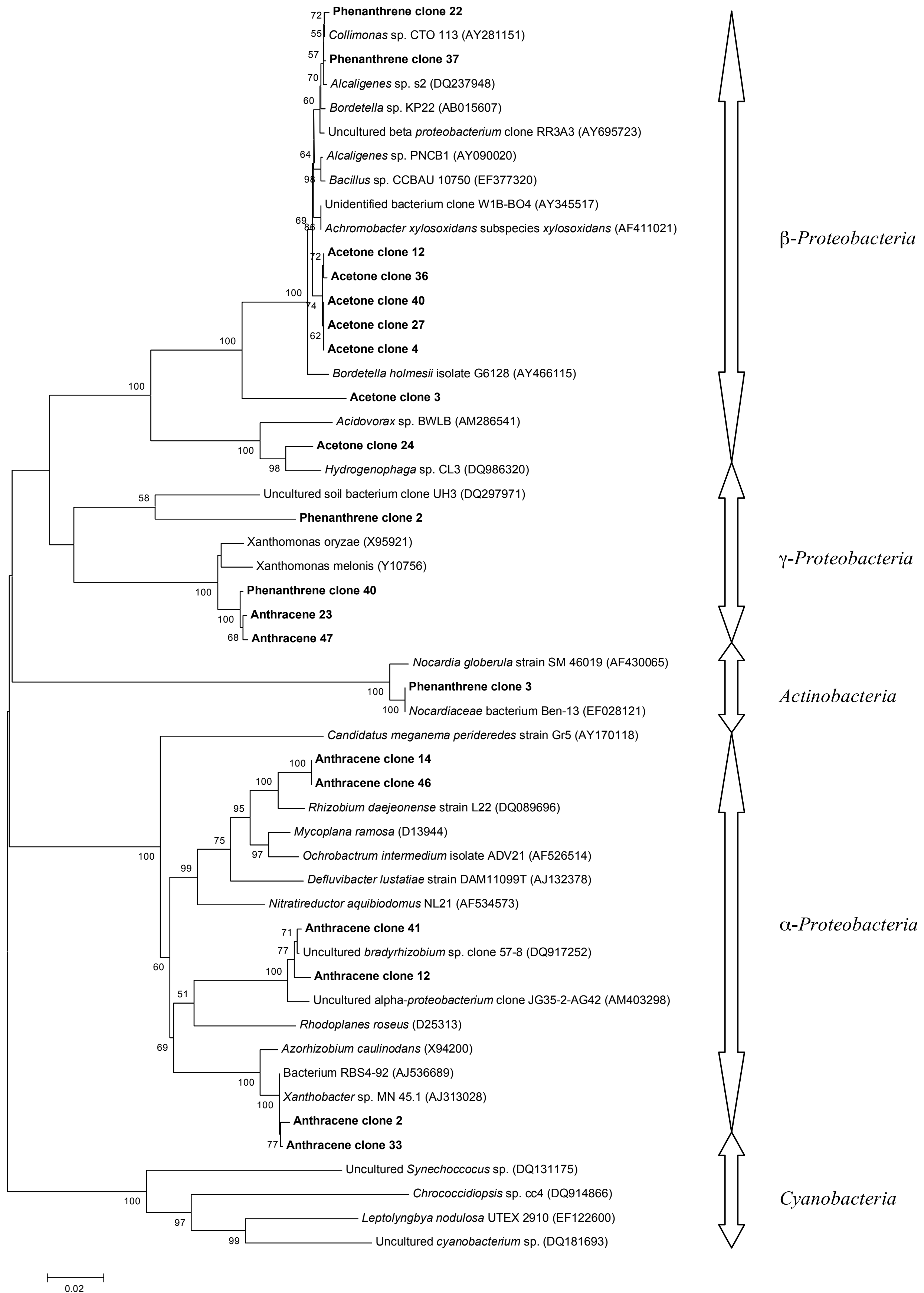

Figure 2.7 Neighbour-joining tree showing phylogenetic relationships of 16S rRNA sequences cloned from the Severn Bridge culture after 37 days enrichment with anthracene/phenanthrene/acetone to closely related sequences from GenBank. Bootstrap

values of >50% are shown. Scale bars indicate Jukes-Cantor distances.

2.4 Discussion

This study assessed the effect of a carrier solvent on the isolation of PAH-degrading bacteria from contaminated sediments within a liquid batch culture enrichment. Enrichment cultures were established on ANT and PHE, in the presence and absence of acetone as a carrier solvent. Although the same sediment sample was used as the bacterial source in all enrichments, different bacterial strains were obtained depending upon the presence of the carrier solvent and the PAH. This is significant when considering methodology for future isolation of PAH-degrading bacteria. In addition, such a finding necessitates review of previously reported PAH-degrading bacterial species that have been isolated and identified from enrichments using a carrier solvent.

Previously reported PAH-degrading bacteria

The most common PAH degrading species identified include *Pseudomonas putida*, *P. aeruginosa, Sphingomonas aromaticivorans, Bacillus pumilis, Mycobacterium gilvum* and *Comamonas testosteroni* (Cerniglia 1992). Reported anthracene-degrading bacteria include: *Beijerinckia* sp., *P. putida, P. fluorescens, P. cepacia, Pseudomonas* spp. (Cerniglia 1992), *Rhodococcus* spp. (Dean-Ross *et al*, 2001) and *Mycobacteria* spp. (Moody 2001).

Comparison of microbial species identified from ANT enrichments in the presence/absence of a carrier solvent

Bacteria isolated and identified (based on the 16S *rRNA* clone library) from the ANT enrichment where acetone was used as a carrier solvent had the greatest homology to two β-*Proteobacteria* (*Achromobacter xylosoxidans* and *Alcaligenes faecalis*), three γ-

Proteobacteria (*P. marginalis, P. fluorescens, Pseudomonas* sp.) and two *Actinobacteria* (*Rhodococcus erythropolis, Rhodococcus* sp.). Bacteria isolated from the ANT crystals enrichment had the greatest homology to six α-*Proteobacteria* (*Xanthobacter* sp., uncultured *Bradyrhizobium* sp., *Rhizobium daejeonense*, *Mycoplana peli*, *Bradyrhizobium* sp., uncultured bacterium) and two γ-*Proteobacteria* (*Xanthomonas oryzae* and *P. boreopolis*). The anthracene enrichment where a carrier solvent was used contained a population with clones that had greatest similarity to species representative of anthracene degraders as identified by Cerniglia (1992) than the clones identified in the anthracene enrichment without a carrier solvent. This highlights the poorly identified anthracene-degrading bacteria within the literature that have been isolated through enrichment cultures with the use of a carrier solvent.

Previously reported PHE-degrading bacterial species

Cerniglia (1992) identified phenanthrene-degrading species as: one α-*Proteobacteria* (*Beijerinckia* sp.); two β-Proteobacteria (*Alcaligenes faecalis, Alcaligenes denitrificans*); five γ-*Proteobacteria* (*Aeromonas* sp., *P. putida*, *P. paucimobilis, Vibrio* sp., *Acinetobacter* sp.); seven *Actinobacteria* (*Arthrobacter polychromogenes,* Micrococcus sp., *Mycobacterium* sp. *, Rhodococcus* sp., *Nocardia* sp., *Streptomyces* sp., *Streptomyces griseus*) and one *Flavobacterium* sp. In addition Andreoni *et al* (2004) identified phenanthrene-degrading species as: three α-*Proteobacteria* (*Methylobacterium* sp., *Rhizobium galegae, Aquamicrobium defluvium);* two β-*Proteobacteria* (*Achromobacter xylosoxidans, Alcaligenes* sp.); one γ-*Proteobacteria* (*Stenotrophomonas acidaminiphilia*) and one *Actinobacterium* (*Rhodococcus aetherovorans*).

Comparison of microbial species identified from PHE enrichments in the

presence/absence of a carrier solvent

The microbial community isolated and identified (based on the 16S *rRNA* clone library) from the PHE enrichment with a carrier solvent present in this study had the greatest homology to three α-*Proteobacteria* (*Bosea minatitlanesis, Bosea* sp., *Xanthobacter* sp.), four γ-*Proteobacteria* (Rhodococcus *erythropolis, Stenotrophomonas maltophilia, Pseudomonas* spp.) three *Actinobacteria* (*Rhodococcus* spp.) and no β-*Proteobacteria*. While the bacteria isolated and identified from the PHE crystals enrichment had the greatest homology to two β-*Proteobacteria* (*Alcaligenes faecalis, Achromobacter xylosoxidans*), two γ-*Proteobacteria* (uncultured soil bacterium, *P. boreopolis*), one *Actinobacterium* (*Norcardiaceae* bacterium) and no α-*Proteobacteria*. The PHE enrichment, which utilized a carrier solvent, contained six clones (67% of the total clone library) with the same species identity as previously reported PHE-degraders, compared to three clones (60% of the total clone library) identified in the PHE enrichment without the use of a carrier solvent. The use of a carrier solvent within enrichment studies where the substrate is not water soluble is a common occurrence (Mueller *et al*, 1997; Dean-Ross *et al*, 2001; Yu *et al*, 2004). There are also studies where the solid form of the substrate within a batch liquid system is utilized (Ahn *et* al, 1999; Andreoni *et al*, 2004; Chadhain *et al*, 2006). Avoiding the use of a carrier solvent circumvents providing an alternative carbon and energy source, however it may result in slower degradation rates due to reduced availability of the substrate to the bacteria.

Alternative experimental procedures

In soil environments, PAHs sorb strongly to organic matter and therefore the bioavailability of PAHs will be greatly reduced. However, studies have shown that specific physiological properties of the microorganisms involved in the degradation of PAHs might enhance the

availability of the compound (Guerin and Boyd, 1997). Techniques have been developed whereby PAHs were supplied sorbed to a solid phase, which allowed enrichment of hydrophobic and adhering PAH-degrading bacteria (Bastiens *et al*, 2000). Additionally, the influence of NAPL phases on PAH degradation have been determined as a modification in the relative frequency of selected phylotypes (Cavalca *et al*, 2008). Such studies attempt to simulate *in situ* conditions in order to isolate potential candidates for bioremediation techniques of PAH-contaminated sites, however, *in situ* conditions can be heterogeneous and extremely complex. Utilisation of these experimental approaches include intrinsic selection biases and the degradative communities subsequently isolated may be ineffective within bioremediation strategies. Enrichment with PAH crystals ensures that the resultant microbial population has been selected on the basis that it can grow on PAHs as the sole carbon source. However, such a degradative population may be unsuccessful within bioremediation techniques due to a lack of specific physiological properties which increase the bioavailability of PAHs sorbed to environmental organic matter.

2.5 Conclusion

This study determined the effect that acetone as a carrier solvent has on the isolation of PAH-degrading bacteria from a sediment sample. α-*Proteobacteria* were identified (based on the 16S *rRNA* clone library) as the major component within the ANT crystal enrichment, whereas γ-*Proteobacteria* comprised the majority of the diversity of bacterial species within the ANT-enrichment when a carrier solvent was present. Within the PHE crystal enrichment, β- and γ-*Proteobacteria* species comprised the majority of the diversity of bacterial species, however α-, γ-*Proteobacteria* and *Actinobacteria* were identified from the PHE enrichment where a carrier solvent was present. The significant shifts in dominant bacterial species in the presence/absence of acetone necessitate the caution required when

using a carrier solvent within an enrichment for hydrophobic xenobiotics. In addition, the use of a carrier solvent within enrichment studies is a common occurrence, however, the findings of this study necessitate a review of previously reported PAH-degrading bacterial species where the use of a carrier solvent has been adopted. Other studies have clearly demonstrated that the form in which the PAH substrate is available to the microbes can significantly affect the resultant degradative population (Bastiens *et al*, 2000; Cavalca *et al*, 2008; Guerin and Boyd, 1997; Johnsen *et al*, 2005) and therefore careful deliberation is required when establishing an enrichment culture to ensure the resultant population will be effective in its defined purpose e.g. bioremediation, bioaugmentation.

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CHAPTER THREE

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

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

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Key words*:* anthracene, phenanthrene, dibenzothiophene, PAHs, PACs, bioremediation.

Abstract

The degradation of polycyclic aromatic compounds (PACs) has been widely studied. Knowledge of the degradation of PACs by microbial populations can be utilised 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 characterised 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.

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 U.S. 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 utilise 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 *et al*, 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 *et al*, 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 *et al*, 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, relative to the other aromatic compounds found in crude oils has also been observed in other studies and contributes to the potential of condensed thiophenes to accumulate 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; Mueller *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 4800 km^3 and its tidal range is the second-highest in the world (Joint Nature Conservation Committee, 2001). Fossil fuel combustion, urban run-off, 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 greater than 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 6800 × 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 pH8.0), then 0.5 ml phenol:chloroform:isoamyl alcohol (pH8.0) (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 seconds to lyse the cells. The tubes were then centrifuged at 11337 × g for 5 minutes. 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 11337 × 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 Units/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 minutes × 1 cycle; 95°C 1 minute, 55°C 1 minute, 72°C 2 minutes × 30 cycles; 72°C 5 minutes × 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 AutoChemiSystem (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'-AGAGTTTGATCCTGGCTCAG-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 were retrieved from GenBank. Sequences were aligned using ClustalX (Chenna *et al*, 2003). Aligned sequences were equivalent to 1514 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 1000 resamplings of the alignments.

Results

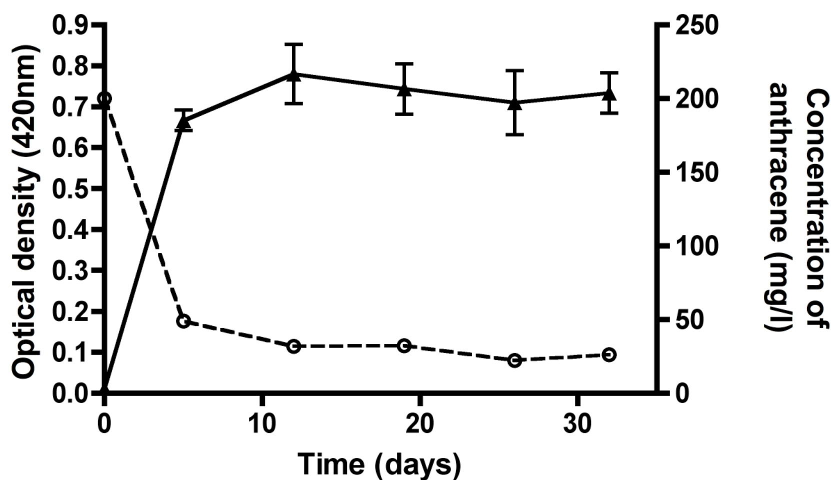
Bacterial growth and PAC degradation

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 (Figure 3.1). 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 measurements of this culture revealed no microbial growth. ANT degradation by the enrichment culture showed a decrease from 200 mg/l to approximately 50 mg/l by day 5

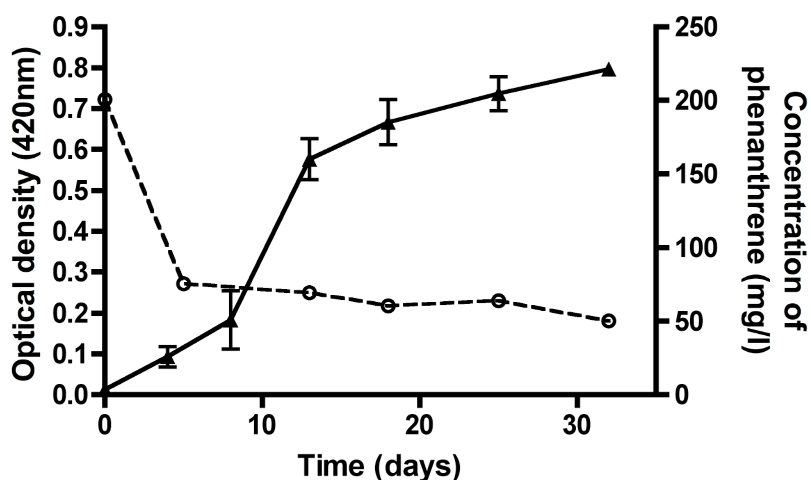
(Figure 3.1A). The ANT enrichment culture showed no lag phase before the onset of exponential growth and reached a maximum optical density of 0.73 (Figure 3.1A). PHE degradation by the enrichment culture showed a decrease from 200 mg/l to 75 mg/l by day 4 (Figure 3.1B). The PHE enrichment culture showed 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 mg/l to 60 mg/l (Figure 3.1C). The DBT-degrading culture showed a lag-phase of 4 days and reached a maximum optical density of 0.615.

Fig. 3.1 Microbial growth (▲) and biodegradation of PACs (**O**) 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.

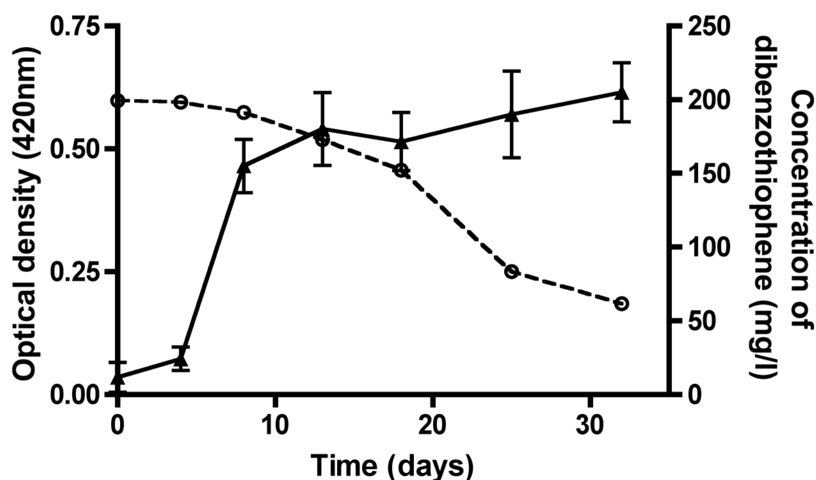
a



b



c

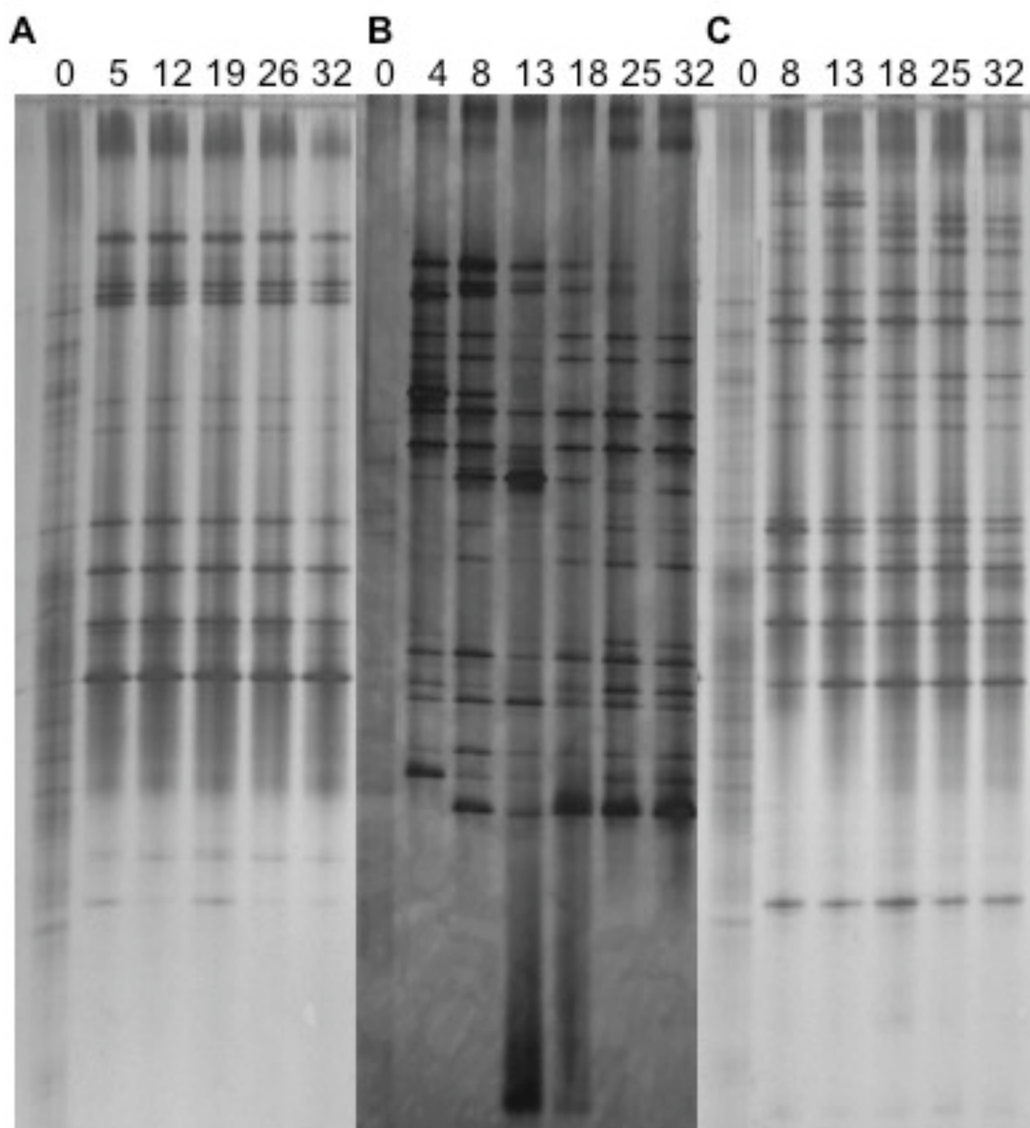


Bacterial community structure

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 (Figure 3.2). 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, 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.

Fig. 3.2 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.



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 3.1). The PHE enrichment contained one clone with closest sequence identity to *Nocardia* sp., one clone as *Alcaligenes* sp., one clone as *Achromobacter xylosoxidans* and one clone as *Pseudomonas* sp. (Table 3.2). 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 3.3).

Table 3.1: 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 3.2: 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 3.3: Closest genera and percentage of identity of the selected clones isolated from the Severn Bridge culture after 37 days enrichment with dibenzothiophene.

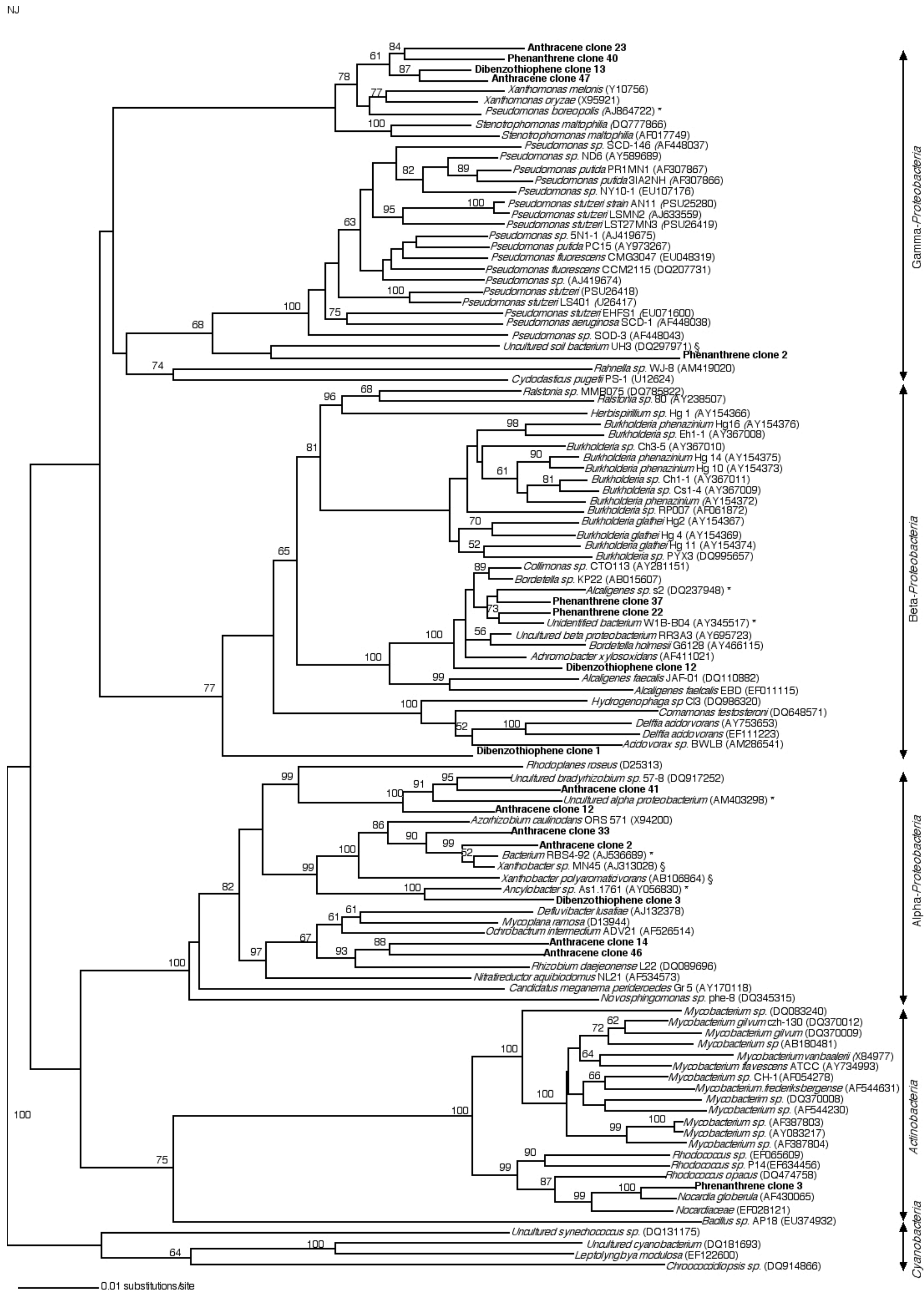
Clone	Genbank accession no	Closest organisms in Genbank database	Similarity[a]
1	AB288556	Uncultured β *Proteobacterium* clone RPS-F2	0.99
3	AY056830	*Ancylobacter* sp. AS1.1761	0.97
12	DQ129607	Uncultured bacterium clone AKIW1148	0.99
13	AJ864722	*Pseudomonas boreopolis* strain S2-s-PMWA-6	0.97

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

Phylogenetic analysis was performed on the ANT/PHE/DBT degradative communities.

All 16S rRNA sequences were aligned and neighbour-joining trees were constructed (Figure 3.3). 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).

Fig. 3.3 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)
Cycloclasticus pugetii PS-1 (U12624)
Ralstonia sp. MMB075 (DQ785822)
Ralstonia sp. 80 (AY238507)
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 faelcalis EBD (EF011115)
Hydrogenophaga sp Cl3 (DQ986320)
Comamonas testosteroni (DQ648571)
Delftia acidovorans (AY753653)
Delftia acidovorans (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 (AY056830) *
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)
Phrenanthrene 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

Discussion

The ability of bacteria to utilise individual PACs as carbon and energy sources has been well documented (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 (figure 3.1A and 3.1B). DBT was degraded at a slower rate (figure 3.1C), 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 was 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 (figure 3.2). 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 (figure 3.3, 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 (Mueller *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 Mueller *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* (figure 3.3). 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 *at 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 (figure 3.3; taxa marked with *). Other studies have found a correlation between bacterial composition and environmental or geographic characteristics, such as salinity, depth and latitude (Cho *et al*, 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 (figure 3.3, 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, 1984).

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 figure 3.3 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 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

There is an urgent need to develop bioremediation techniques for polluted sites 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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CHAPTER FOUR

Phylogenetic analysis reveals multiple lateral transfer events of PAH dioxygenase gene between different classes of *Proteobacteria* but limited between *Actinobacteria* and *Proteobacteria*

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Phylogenetic analysis reveals multiple lateral transfer events of PAH dioxygenase gene between different classes of *Proteobacteria* but limited between *Actinobacteria* and *Proteobacteria*

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous recalcitrant environmental pollutants with potentially deleterious effects on human health. Knowledge of PAH degradation can be utilised in the remediation of contaminated sites. PAH-dioxygenases catalyse the first step in the aerobic degradation of PAHs and are generally found on self-transmissible plasmids. To investigate the evolution of PAH degradation, this study determined the congruency of tree topology for PAH-dioxygenases and the corresponding 16S rRNA from PAH-degrading species. Phylogenetic analysis was performed on the large Fe-S subunit (α subunit) of PAH-dioxygenase sequences from reported PAH-degrading species (GenBank). Comparative analysis of the α subunit sequences of PAH dioxygenases produced a tree topology that is partially inconsistent with the corresponding 16S rRNA tree. The findings suggest that the α subunit fragment may have been laterally transferred between classes of the *Proteobacteria*. The presence of identical regions within the α subunit sequences of PAH dioxygenases within taxonomically diverse species provides additional evidence for lateral transfer. It was found that the α subunit fragment from *Actinobacteria* constituted a separate cluster to the *Proteobacteria*. This indicates little lateral transfer between the *Proteobacteria* and *Actinobacteria,* despite PAH-degradative abilities being present in both groups. Additional evidence for little lateral transfer between *Actinobacteria* and *Proteobacteria* arises from the

identification of indels that are unique to the *Actinobacteria.* This may indicate that the PAH dioxygenase evolved early, prior to the separation of the *Actinobacteria* and the *Proteobacteria* and has subsequently undergone divergent evolution to result in different homologs. We reveal the extent to which PAH dioxygenase can be transferred between bacterial species, indicating the adaptation of microbial communities to degrade environmental pollutants; the understanding of which contributes to the development of bioremediation strategies.

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.

Genetic control of naphthalene biodegradation

The naphthalene catabolic genes within the well-studied *Pseudomonas putida* G7 strain are organised into three operons on the 83-kb NAH7 plasmid: one encoding the upper-pathway enzymes involved in the conversion of naphthalene to salicylate, the second encoding the lower pathway enzymes responsible for converting salicylate into the TCA cycle intermediate and the third encoding the regulatory protein NahR (Habe and Omori, 2003).

Homology of naphthalene degradative genes

Nucleotide sequences of genes encoding the naphthalene degradative enzymes from several *Pseudomonas* strains have been reported and are shown to have high sequence homology (about 90%) and similar gene organisation (Habe and Omori, 2003). High

sequence similarity of degradative genes could imply that lateral transfer has occurred between these different species. However, the nucleotide sequence homology and the organization of isofunctional clusters of genes cloned from genera *Burkholderia, Comamonas, Ralstonia* and *Sphingomonas* are not so conserved with respect to the *nah* genes (Zocca *et al*, 2004). This may indicate limited lateral transfer between these genera and the Pseudomonads. Additionally, it has been revealed that dioxygenases of Gram-positive organisms are only distantly related to PAH dioxygenases of Gram-negative strains. This may indicate that little horizontal transfer of these degradative genes has occurred between the Gram-positive and Gram-negative bacteria.

Naphthalene dioxygenase and functionally important amino acid residues

Naphthalene dioxygenase (NDO) catalyses the first step in the aerobic degradation of naphthalene by adding both atoms of oxygen to the aromatic nucleus, forming *cis*-naphthalene dihydrodiol (Ni Chadhain *et al*, 2006). The NDO system consists of three components; an iron-sulfur flavoprotein reductase, an iron-sulfur ferredoxin and the catalytic oxygenase component (Moser and Stahl, 2001). The oxygenase is composed of large and small subunits (α and β) that are arranged as an $\alpha_3\beta_3$ hexamer (Ni Chadhain *et al*, 2006). The oxygenase α subunits are responsible for determining the substrate specificities of NDO (Parales *et al*, 2000). Therefore, this study will focus on the Fe-S center of the dioxygenase α subunit of various PAH-dioxygenase. The role of active-site residues in NDO have been investigated and 15 amino acid residues have been identified with putative importance for functionality (Parales *et al*, 1999, 2000).

Comparative analysis of functional gene and 16S rRNA sequences to investigate the evolution of functional genes

Determining the congruency of the tree topologies for functional genes and the corresponding 16S rRNA from degradative populations can suggest mechanisms for the evolution of functional genes (Friedrich, 2002; Herrick *et al*, 1997; Klein *et al*, 2001; Wilson *et al*, 2003). To investigate the evolution of PAH degradation, this study determined the congruency of tree topologies for the α subunit of PAH dioxygenases and the corresponding 16S rRNA from PAH-degrading species. The 16S rRNA gene was selected based on the following characteristics of the gene as outlined by Woese: (i) there is no evidence of horizontal transfer of the 16S rRNA gene or portions thereof (although some reports suggest otherwise [Eardly *et al*, 1995, 2004; Sneath *et al*, 1993]); (ii) it is highly conserved, with significant sequence identity retained even across domain and kingdom boundaries; and (iii) its phylogeny is considered representative of the phylogeny of the organism possessing it (Anzai *et al*, 2000; Herrick *et al*, 1997).

Phylogenetic analysis was performed on the α subunit sequences of PAH-dioxygenases from previously reported PAH-degrading species (Genbank). Comparative analysis of PAH-dioxygenase sequences from the *Proteobacteria* produced a tree topology that is inconsistent with the corresponding 16S rRNA tree and may indicate lateral transfer of the α subunit fragment between the different classes of this phylum. The presence of identical regions within the α subunit sequences in taxonomically diverse species provides additional evidence for lateral transfer of the PAH-dioxygenase gene. To further investigate the occurrence of lateral transfer, this study determined the conformity of the α subunit sequences with the putative functionally important amino acid residues (Parales *et al*, 1999; 2000). The

outcome of this research has important implication for the development of bioremediation techniques for the clear up of PAH-contaminated environments.

Materials and Method

Sequence alignment and phylogenetic analysis

Sequences were aligned using the program Clustal X (Thompson *et al*, 1997). 16S rRNA aligned sequences were equivalent to 1514 bp of 16S rRNA sequence of *Escherichia coli* strain SFC6. The α subunit fragment alignment included 500 amino acid positions, some partial sequences of length 200 amino acid residues were also analysed.

Phylogenetic analysis was performed using the program PAUP 4.0 (Swofford, 2000) and data were analysed using distance and neighbour-joining methods. Distance and neighbour-joining analysis was implemented using the default options of PAUP. Analyses were performed separately for each set of gene sequences. For all analyses, relative bootstrap support was estimated using 1000 replicates for each gene.

The principle phylogenetic analysis of the α subunit of PAH dioxygenase data included 138 unique sequences (i.e. all identical sequences were excluded); 30 sequences originated from *Actinobacteria* taxa, 15 sequences from α-*Proteobacteria* taxa, 23 sequences from β- *Proteobacteria* taxa and 70 sequences from γ-*Proteobacteria* taxa. The phylogenetic analysis of 16S rRNA data included 88 unique sequences; 20 sequences originated from *Actinobacteria* taxa, three sequences from α-*Proteobacteria* taxa, 21 sequences from β- *Proteobacteria* taxa and 34 sequences

from γ-*Proteobacteria* taxa while three were from outgroup taxa, comprising *Cyanobacteria*. Sequences were obtained from GenBank.

Results

α subunit of PAH-dioxygenase amino acid phylogeny

Phylogenetic analysis was performed on alignments of previously reported α subunit amino acid sequences of PAH dioxygenases (figure 4.1). For comparison, trees were calculated from the 16S rRNA genes for the corresponding species (figure 4.2). Two major lineages have been identified in the reported α subunit-based analysis: *Actinobacteria* and *Proteobacteria;* this is consistent with the 16S rRNA-based analysis. Major incongruencies were found between the reported α subunit- and the 16S rRNA-based analysis for the α, β, and γ-*Proteobacteria*. The 16S rRNA tree indicates that the *Actinobacteria* are most distantly related to the *Proteobacteria.* Of the *Proteobacteria,* the β- and γ-*Proteobacteria* are most closely related.

Incongruence between the 16S rRNA phylogeny and α subunit phylogeny within the α-*Proteobacteria*

In contrast to relationships inferred using the rRNA sequences, the α-*Proteobacteria* are not monophyletic in the α subunit tree (figure 4.1). The α-*Proteobacteria* have formed a group which is most closely related to the *Cycloclasticus* spp., belonging to the γ-*Proteobacteria* (see Alpha-*Proteobacteria* group on figure 4.1). Four *Sphingomonas* spp. (CAG17579, AAZ95271, ABC66285 and BAA90523) have formed a group with two *Terrabacter* spp. (AAZ38357 and ABA87074) and with a

Mycobacterium sp. SNP11 (ABK27713). Interestingly, *Sphingomonas* sp. A4 (BAD34447) has clustered most closely to γ- and β-*Proteobacteria* groups.

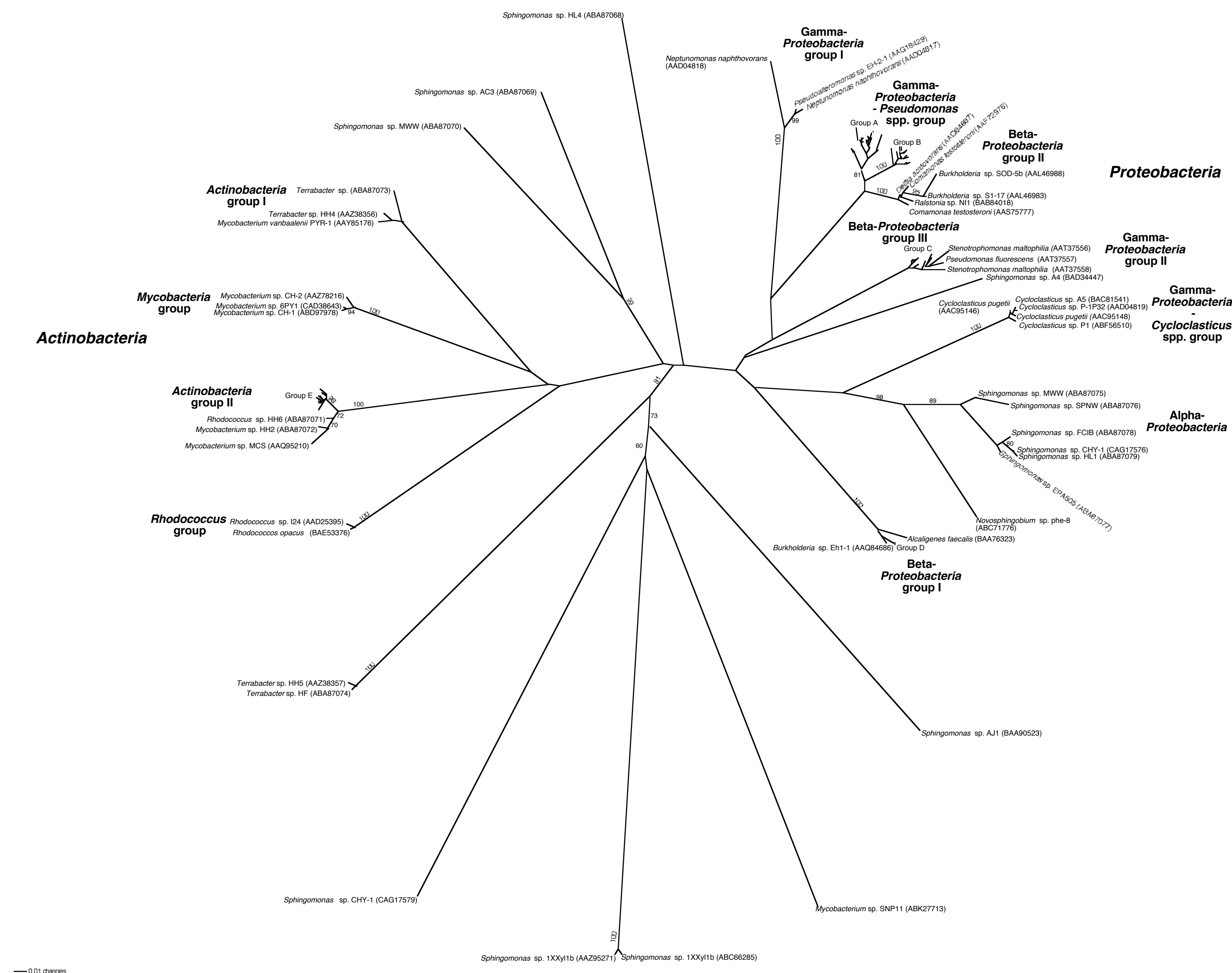

Figure 4.1: Neighbour-joining tree constructed using PAUP 4.0 showing the phylogenetic relationship of α subunit of PAH dioxygenase from PAH-degrading species reported on GenBank. Bootstrap percentages of 50% or more are indicated. Defined clusters have been identified as: *Actinobacteria* group I; *Actinobacteria* group II; *Mycobacteria* group; *Rhodococcus* group; γ-*Proteobacteria* group I; γ-*Proteobacteria* group II; γ-*Proteobacteria-Pseudomonas* spp. group; γ-*Proteobacteria-Cycloclasticus* spp. group; β-*Proteobacteria* group I; β-*Proteobacteria* group II; α-*Proteobacteria* group. Species names and GenBank accession numbers of taxa within groups A, B, C, D and E are listed in table 4.1.

Group A	GenBank accession no.
Pseudomonas sp. LCY14	AAV33334
Pseudomonas sp. 8IDINH	AAG25693
Pseudomonas sp. 5N1-1	CAD42906
Pseudomonas fluorescens	AAR07510
Pseudomonas sp. 4N1-3	CAD42904
Pseudomonas sp. 4N4-1	CAD42905
Pseudomonas fluorescens	AAR07508
Pseudomonas sp. 4N1-2	CAD42903
Pseudomonas fluorescens	AAR07509
Pseudomonas putida	AAG25696
Pseudomonas sp. LZT5	ABA29806
Pseudomonas aeruginosa	AAL46984
Pseudomonas sp. CY13	AAV33335
Pseudomonas sp. 19IIDNH	AAG25686
Pseudomonas balearica	AAG25680
Pseudomonas stutzeri	AAG25684
Pseudomonas stutzeri	AAG25687
Pseudomonas stutzeri	AAO45605
Pseudomonas stutzeri	AAO45604
Marinobacter sp. NCE312	AAG18427
Pseudomonas sp. LSMN7	AAG25691
Pseudomonas sp. SOD-3	AAL46987
Pseudomonas sp. PR3MN2	AAG25694
Pseudomonas putida	BAA20391
Pseudomonas sp. PZT2	AAV33341
Pseudomonas putida	NP_863072
Pseudomonas putida	AAG25692
Pseudomonas sp.	AAA92141
Rahnella sp. CY14	AAV33333
Pseudomonas sp. LZT1	ABA29805
Pseudomonas sp. SCD-14b	AAL46985
Pseudomonas putida	AAO64274
Pseudomonas fluorescens	AAM94035
Pseudomonas putida	AAB47591
Pseudomonas sp. LCY11	AAV33338
Pseudomonas sp. LCY18	AAV33340
Pseudomonas aeruginosa	AAL46982
Pseudomonas sp. SCD-3a	AAL46986
Pseudomonas sp. CY24	AAV33337
Pseudomonas sp. PZT1	AAV33336
Pseudomonas sp. ND6	NP_943188
Pseudomonas sp. LCY16	AAV33339
Pseudomonas sp. ND6	AAP44288
Pseudomonas putida	YP_534822
Pseudomonas putida	BAE92156
Pseudomonas putida	AAA25902
Pseudomonas fluorescens	AAL07262

Group B	GenBank accession no.
Pseudomonas putida	AAG25690
Pseudomonas stutzeri	AAG25682
Pseudomonas stutzeri	AAD02136
Pseudomonas stutzeri	AAG25695
Pseudomonas stutzeri	AAG25679
Pseudomonas stutzeri	AAG25683
Pseudomonas stutzeri	AAG25685
Pseudomonas balearica	AAG25681
Pseudomonas balearica	AAG25689
Pseudomonas balearica	AAG25688
Pseudomonas sp. 2N1-1	CAD42902
Pseudomonas aeruginosa	BAA12240
Group C	
Burkholderia phenazinium	AAN74950
Burkholderia phenazinium	AAN74947
Burkholderia phenazinium	AAN74949
Burkholderia phenazinium	AAN74946
Burkholderia glathei	AAN74948
Burkholderia glathei	AAN74945
Burkholderia glathei	AAN74944
Burkholderia sp. RP007	AAD09872
Delftia acidovorans	AAQ84688
Ralstonia sp. PJ531	BAB84024
Herbaspirillum sp. Hg 1	AAN74943
Burkholderia sp. VUN10,013	AAT37559
Group D	
Burkholderia sp. Ch1-1	AAQ84683
Burkholderia sp. Cs1-4	AAQ84685
Burkholderia sp. Ch3-5	AAQ84684
Group E	
Mycobacterium gilvum	AAN78316
Mycobacterium gilvum	ABG02224
Mycobacterium flavescens	AAN78312
M. frederiksbergense	AAN78314
Mycobacterium vanbaalenii	AAT51751
Mycobacterium sp. MHP-1	BAD20297
Mycobacterium sp. 6PY1	CAD38647
Mycobacterium sp. S65	AAQ12023
Mycobacterium sp. CH-1	ABD97969
Mycobacterium sp. JLS	AAQ95206
Mycobacterium sp. czh-8	ABG02223
Mycobacterium sp. KMS	AAQ95208
Mycobacterium sp. PYR-1	AAF75991
Mycobacterium sp. CH-2	AAZ78224

Table 4.1: Species names and accession numbers for taxa within groups A, B, C, D and E from the α-subunit phylogeny (figure 4.1).

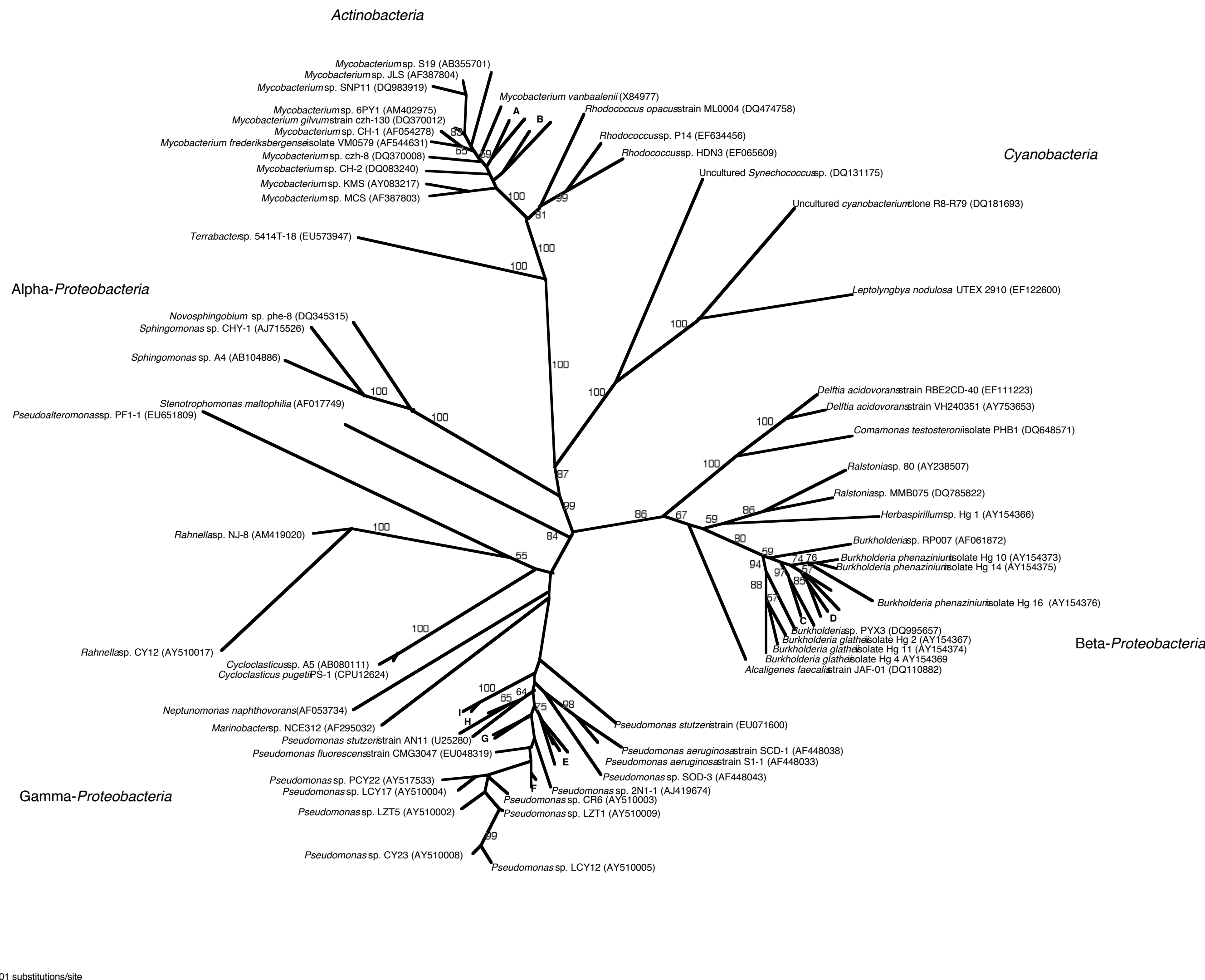

Figure 4.2: Neighbour-joining tree constructed using PAUP 4.0 showing the phylogenetic relationship of the 16S rRNA sequences from PAH-degrading species reported on GenBank. Bootstrap percentages of 50% or more are indicated. The α-, β- and γ-subclasses of the *Proteobacteria*, the *Actinobacteria* and *Cyanobacteria* groups have been indicated. Species names and GenBank accession numbers of taxa within groups A to I are listed in table 4.2.

Group A	GenBank accession no.
Mycobacterium sp. MHP-1	AB180481
Mycobacterium gilvum strain czh-101	DQ370009
Group B	**GenBank accession no.**
Mycobacterium flavescens	AY734993
Mycobacterium sp. S65	AF544230
Group C	**GenBank accession no.**
Burkholderia sp. SOD-5b	AF448044
Burkholderia sp. Cs1-4	AY367009
Group D	**GenBank accession no.**
Burkholderia sp. Ch3-5	AY367010
Burkholderia phenazinium Hg 8	AY154372
Burkholderia sp. Ch1-1	AY367011
Burkholderia sp. Eh1-1	AY367008
Group E	**GenBank accession no.**
Pseudomonas putida PR1MN1	AF307867
Pseudomonas sp. NY10-1	EU107176
Pseudomonas sp. ND6	AY589689
Pseudomonas putida 3IA2NH	AF307866
Group F	**GenBank accession no.**
Pseudomonas sp. 5N1-1	AJ419675
Pseudomonas fluorescens	DQ207731
Group G	**GenBank accession no.**
Pseudomonas sp. SCD-14b	AF448037
Pseudomonas putida strain PC15	AY973267
Group H	**GenBank accession no.**
Pseudomonas stutzeri	AJ633559
Pseudomonas stutzeri strain ST27MN3	U26419
Group I	**GenBank accession no.**
Pseudomonas stutzeri strain LS401	U26417
Pseudomonas stutzeri strain SP1402	U26418

Table 4.2: Species names and accession numbers for taxa within groups A to I from the 16S rRNA phylogeny (figure 4.2).

Incongruence between the 16S rRNA phylogeny and α subunit phylogeny within the γ-*Proteobacteria*

Incongruency was found between the α subunit- and the 16S rRNA-based analysis for the γ-*Proteobacteria*. In contrast to relationships inferred using the rRNA, the γ-*Proteobacteria* is not monophyletic in the α subunit tree. A majority of the Pseudomonads form a single clade with *Rahnella* sp. (AAV3333) and *Marinobacter* sp. NCE312 (AAG18427) which is closely related to β-*Proteobacteria* group II (figure 4.1). An additional three separate γ-*Proteobacteria* groups were formed: *Cycloclasticus* spp. (see Gamma-*Proteobacteria* – *Cycloclasticus* spp. group on figure

4.1) has formed a separated grouping, clustering more closely to α-*Proteobacteria* than to the Pseudomonads. *Neptunomonas* spp. (AAD04818, AAD04817) and *Pseudoalteromonas* sp. EH-2-1 (AAG18429) have formed a separated grouping (see Gamma-*Proteobacteria* group I on figure 4.1). *Stenotrophomonas* spp. (AAT37556, AAT37558) and *Pseudomonas fluorescens* (AAT37557) form a separated grouping, together with β-*Proteobacteria* group III (see Gamma-*Proteobacteria* group II on figure 4.1).

Incongruence between the 16S rRNA phylogeny and α subunit phylogeny within the β-*Proteobacteria*

In contrast to relationships inferred using the rRNA, the β-*Proteobacteria* are not monophyletic in the α subunit phylogeny. The β-*Proteobacteria* have formed three different groupings: β-*Proteobacteria* group I with *Alcaligenes faecalis* (BAA76323); β-*Proteobacteria* group II with the *Pseudomonas* spp. group; β-*Proteobacteria* group III with *Stenotrophomonas* spp. (AAT37556, AAT37558) and *Pseudomonas fluorescens* (AAT37557) (figure 4.1).

Incongruence between the 16S rRNA phylogeny and α subunit phylogeny within the *Actinobacteria*

The α subunit-based analysis has clustered a majority of the *Actinobacteria* as a separate group (see *Actinobacteria* group in figure 4.1) which is consistent with 16S rRNA-based analysis. However, *Terrabacter* spp. HH5 and HF (AAZ38357, ABA87074) have formed a slightly more distant separate grouping with *Mycobacterium* sp. SNP11 (ABK27713) and *Sphingomonas* spp.

Within the *Actinobacteria* group there is incongruency between the α subunit-based analysis and the 16S rRNA-based analysis. Four major groups have formed: *Actinobacteria* group I; *Mycobacteria* group; *Actinobacteria* group II and *Rhodococcus* group. α subunit fragments isolated from *Mycobacteria* spp. appear to be the most promiscuous as there are *Mycobacteria* α subunit genes present within the *Actinobacteria* group I, *Mycobacteria* group and *Actinobacteria* group II. Interestingly, two main *Mycobacteria* groups have formed: one containing only *Mycobacteria* spp. (*Mycobacteria* group) the other consisting mostly of *Mycobacteria* and *Rhodococcus* sp. HH2 (*Actinobacteria group* II). The 16S rRNA-based analysis clustered *Mycobacteria* most closely with *Rhodococcus* whilst *Terrabacter* is more distantly related to the other *Actinobacteria*; the α subunit-based analysis is in congruence with this as *Rhodococcus* sp. HH6 (ABA87071) has clustered with *Mycobacteria* spp. in the *Actinobacteria* group II. However, contradictory to the 16S rRNA analysis, the α subunit phylogeny has clustered two *Rhodococcus* taxa (*Rhodococcus* group) most distantly to the other *Actinobacteria*. Additional incongruence can be seen between the 16S rRNA phylogeny and the α subunit phylogeny within the *Terrabacter* taxa, whereby two *Terrabacter* spp. (ABA87073 and AAZ38356) have formed a separate group with *Mycobacteria vanbaalenii* PYR-1 (AAY85176; see *Actinobacteria* group I, figure 1).

Conformity to putative functionally important amino acid residues

A possible interpretation for the topological conflicts between the α subunit - and 16S rRNA-based analyses is lateral transfer of the dioxygenase genes. The amino acid alignment for α subunit were analysed for evidence of lateral transfer of the gene among the α-, β-, γ-*Proteobacteria*. Parales *et al* (2000) proposed 15 amino acid residues with putative importance for functionality within the α subunit of the

dioxygenases. Table 4.3 shows the number of sequences within each bacterial grouping (as determined from phylogenetic analysis, see figure 4.1) that contain the putative important active site residues. The *Pseudomonas* spp. group, γ-*Proteobacteria* group II and β-*Proteobacteria* group III contain no residue substitutions. β-*Proteobacteria* group I contains one substitution at position 358. γ-*Proteobacteria* group I and β-*Proteobacteria* group II contain one substitution at position 260. *Cycloclasticus* spp. group contains two substitutions at positions 260 and 358. α-*Proteobacteria* group contains three substitutions at positions 201, 260 and 358. *Actinobacteria* group contains four substitutions at positions 83, 260, 358 and 366. Table 4.3 shows the amino acids that have been substituted at the positions of the putative important active site residues. Taxa that have not clustered in defined groups in the α subunit phylogeny (figure 4.1) are collectively referred to as "other" in table 4.3.

Homology in α subunit amino acid sequences of the PAH dioxygenases

Additional evidence for lateral transfer of the α subunit of the PAH-dioxygenase gene between *Pseudomonas* spp. and species within β-*Proteobacteria* group II arises from the identification of identical regions of the α subunit fragment (figure 4.3). There are positions that contain variable residues within the sequences, however in most cases these positions are replaced with only two different residues.

	Total	C81	H83	C101	H104	N201	F202	D205	H208	H213	V260	W316	F352	W358	D362	M366
	Number of sequences containing the amino acid residues															
***Actinobacteria* gp**	25	22	21 (1F)	25	25	25	25	25	25	25	0 (25AMFL)	22	22	0(22F)	22	0 (22FW)
Gamma-*Proteobacteria* (*Pseudomonas* spp. gp)	59	42	42	42	42	59	59	59	59	59	59	47	14	14	14	14
Beta-*Proteobacteria* gp 2	6	3	3	4	4	6	6	6	6	6	4 (2I)	6	1	1	1	1
Gamma-*Proteobacteria* gp 1	3	0	0	0	0	3	3	3	3	3	0 (3I)	0	0	0	0	0
Beta-*Proteobacteria* gp 3	12	11	12	12	12	12	12	12	12	12	12	10	8	1	1	1
Gamma-*Proteobacteria* gp 2	3	1	3	3	3	3	3	3	3	3	3	0	0	0	0	0
Gamma-*Proteobacteria* (*Cycloclasticus* spp. gp)	5	2	2	2	2	5	5	5	5	5	0 (5I)	2	2	0 (2L)	2	2
Alpha-*Proteobacteria* gp	7	1	1	2	4	3 (1Y)	4	4	4	4	0 (4I)	4	1	0 (1L)	1	1
Beta-*Proteobacteria* gp1	5	5	5	5	5	5	5	5	5	5	5	5	1	0 (1L)	1	1
Other	11	1 (3MPD)	1 (3DLG)	4 (4SLTH)	4 (4GAQG)	3 (5LNGF)	5 (3VLY)	5 (2PL)	4 (4NR)	3 (5MIRY)	1 (6AL)	1 (1F)	1	1	1	1

Table 4.3: Number of sequences that contain the putative important active site residues. The values in the parentheses indicate the number of substitutions at the active site amino acid positions. The letters in the parentheses correspond to the amino acids at the substitutions.

There is additionally evidence for lateral transfer of the α subunit of the PAH-dioxygenase gene between the species within γ-*Proteobacteria* group II and β-*Proteobacteria* III from identical regions in the α subunit fragment (figure 4.4). A majority of the positions that contain variable residues within the sequences contain only two different residues.

```
Microorganisms                                    Amino acid positions
Pseudomonas spp.     81        90         100       110       120              189        200       210       220
AAG25694    GSIRAFLNVCRHRGKTLVS-VEAGNAKGFVCSYHGWGFGSNGELQSVPFEKDLYGESLN   VVIKANWKAPAENFVGDAYHVGWTHASSLRSGESI
AAG25693    GSIRAFLNVCRHRGKTLVS-VEAGNAKGFVCSYHGWGFGSNGELQSVPFEKDLYGESLN   VVIKANWKAPAENFVGDAYHVGWTHASSLRSGESI
AAB47591    GSIRAFLNVCRHRGKTLVH-VEAGNAKGFVCSYHGWGFGSNGELQSVPFEKDLYGESLN   VVIKANWKAPAENFVGDAYHVGWTHASSLRSGESI
NP_943188   GSIRAFLNVCRHRGKTLVH-VEAGNAKGFVCSYHGWGFGSNGELQSVPFEKDLYGESLN   VVIKANWKAPAENFVGDAYHVGWTHASSLRSGESI
β-proteobacteria spp.
BAB84018    ------------------------NAKGFFCSYHGWGFGSNGELQGVPFEKELYGDAIK   VVIKANWKAPAENFVGDAYHVGWTHASSLRSGQSI
AAQ84687    GSVRAFLNVCRHRGKTLVH-AEAGNAKGFVCSYHGWGFGSNGELQSVPFEKELYGDAIK   VVIKANWKAPAENFVGDAYHVGWTHASSLRSGQSI
AAL46988    ---------------------------------------PTANCKSVPFEKDLYGDSIK   VVIKANWKAPAENFVGDAYHVGWTHAASLRSGQSI
AAL46983    ---------------------------------------SNGELQSVPFEKDLYGDSIK   VVIKANWKAPAENFVGDAYHVGWTHAASLRSGQSI
AAF72976    GSVRAFLNVCRHRGKTLVH-AEAGNAKGFVCSYHGWGFGSNGELQSVPFEKELYGDAIK   VVIKANWKAPAENFVGDAYHVGWTHAASLRSGQSI
AAS75777    GSVRAFLNVCRHRGKTLVH-AEAGNAKGFVCSYHGWGFGSNGELQSVPFEKELYGDAIK   VVIKANWKAPAENFVGDAYHVGWTHASSLRTGQSI
Sphingomonas sp. A4
BAD34447    GTVKAFLNVCRHRGARVCP-VEAGNRRAFVCNYHGWSYAADGSLAAIPFEKELYGGKID   SIVHCNWKAPTENFVGDAYHIGWTHASSLAASRSIF

Pseudomonas spp.     240        250       260         270       280       290       300       310
AAG25694    AL-PPEGAGLQTTSKYGSGMGVLWDGYSGVHSAD--LVPELMAFGGAKQERLNKEIGDVRARIYRSHLNCTVFPNNSMLTCSG
AAG25693    AL-PPEGAGLQMTSKYGSGMGVLWDGYSGVHSAD--LVPELMAFGGAKQEGLNKEIGDVRARIYRSHLNCTVFPNNSMLTCSG
AAB47591    AL-PPEGAGLQMTSKYGSGMGVLWDGYSGVHSAD--LVPELMAFGGAKQERLNKEIGDVRARIYRSHLNCTVFPNNSMLTCSG
NP_943188   AL-PPEGAGLQMTSKYGSGIGVLWDVYSGVHSAD--LVPELMAFGGAKQERLNKEIGDVRARIYRSHLNCTVFPNNSMLTCSG
β-proteobacteria spp.
BAB84018    ML-PPEGAGLQMTSKYGSGMGVLWDAYSGVHSAD--LVPEMMAFGGAKQEKLAKEIGDVRARIYRSHLNCTVFPNNSILTCSG
AAQ84687    ML-PPEGAGLQMTSKYGSGMGVLWDAYSGVHSAD--LVPEMMAFGGAKQEKLAKEIGDVRARIYRSHLNCTVFPNNSILTCSG
AAL46988    ML-PPEGAGLQMTSKYGSGMGVLWDAYSGVHSAD--LVPEMMAFGGAKQEKLAKEIGDVRARIYRSHLNCTVFPNNSILTCSG
AAL46983    ML-PPEGAGLQMTSKYGSGMGVLWDAYSGVHSAD--LVPEMMAFGGAKQEKLAKEIGDVRARIYRSHLNCTVFPNNSILTCSG
AAF72976    ML-PPEGAGLQMTSKYGSGMGVLWDAYSGIHSAD--LVPEMMAFGGAKQEKLAKEIGDVRARIYRSHLNCTVFPNNSILTCSG
AAS75777    ML-PPEGAGLQMTSKYGSGMGVLWDGYSGIHSAD--LVPDMMAFGGAKQEKLAKEIGDVRARIYRSHLNCTVFPNNSILTCSG
Sphingomonas sp. A4
BAD34447    ML-PPAGAGAQIATRFGHGLGILYDVNPGVHTAQ--TAEKILAWQATKKDKIAEKYGELKARFYGSHLNGSLFPNVSYLWGTN
```

Figure 4.3: Amino acid alignment of the α subunit of PAH dioxygenases from *Pseudomonas* spp. and members from the β-*Proteobacteria* group II. Amino acid residues that are homologous have been highlighted.

```
Microorganisms                                   Amino Acid positions

γ-proteobacteria group 2        200         210         220         230          240         250         260         270         280
AAT37557 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTRHGHGIGALYDVYAGVHDNEELMAWGLAKEQ
AAT37556 PGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTRHGHGIGALYDVYAGVHDNEELMAWGLAKEQ
AAT37558 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTRHGHGIGALYDVYAGVHDNEELMAWGLAKE Q
β-proteobacteria group 3
AAT37559 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTRHGHGIGALYDVYAGVHDNEELMAWGLAKEQ
BAB84024 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTRHGHGIGALYDVYAGVHDNEELMAWGLAKEQ
AAQ84688 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTRHGHGIGALYDVYAGVHDNE-----------
AAD09872 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTRHGHGIGALYDVYAGVHDNEELMAWGLAKEQ
AAN74949 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQHGHGIGALYDVYAGVHDNEELMAWGVAKEQ
AAN74950 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQHGHGIGALYDVYAGVHDNEELMAWGVAKEQ
AAN74945 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQHGHGIGALYDVYAGVHDNEELMAWGVAKEQ
AAN74944 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQHGHGIGALYDVYAGVHDNEELMAWGVAKEQ
AAN74943 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQHGHGIGALYDVYAGVHDNEELMAWGVAKE Q
AAN74948 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQHGHGIGALYDVYAGVHDNEDLMAWGLAKEQ
AAN74946 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQHGHGIGALYDVYAGVHDNEDLMAWGLAKEQ
AAN74947 AGG-GIELVGPPAR---CFIEANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQHGHGIGALYDVYAGVHNNEDLMAWGLAKEQ

γ-proteobacteria group 2      300
AAT37557 VLKEKIGPIRARLYRSHLNGTMFPNS
AAT37556 VLKEKIGPIRARLYRSHLNGTIFPNT
AAT37558 VLKEKIGPIRARLYRSHLNGTMSPS-
β-proteobacteria group 3
AAT37559 VLKEKIGSIRARLYRSHLNGTIFPNT
BAB84024 -------------------------- -
AAQ84688 VLKEKIGPIRARLYRSHLNGTIFPN T
AAD09872 VLKDKIGPICARLYRSHLNETIFPNT
AAN74949 VLKDKIGPIRARLYRSHLNGTIFPNT
AAN74950 VLKDKIGPIRARLYRSHLNGTIFPNT
AAN74945 VLKDKIGPIRARLYRSHLNGTIFPNT
AAN74944 VLKDKIGPIRARLYRSHLNGTIFPNT
AAN74943 VLKDKIGPIRARLYRSHLNGTIFPNT
AAN74948 VLKDKIGPIRARLYRSHLNGTIFPNT
```

Figure 4.4: Amino acid alignment of the α subunit of PAH dioxygenases from members of the γ-*Proteobacteria* group II and members from β-*Proteobacteria* group III. Amino acid residues that are homologous have been highlighted.

Additional evidence for lateral transfer of the α subunit of the PAH-dioxygenase gene between the species within α-*Proteobacteria* group and the γ-*Proteobacteria*-*Cycloclasticus* spp. group arises from the identification of identical regions of the α subunit fragment (figure 4.5).

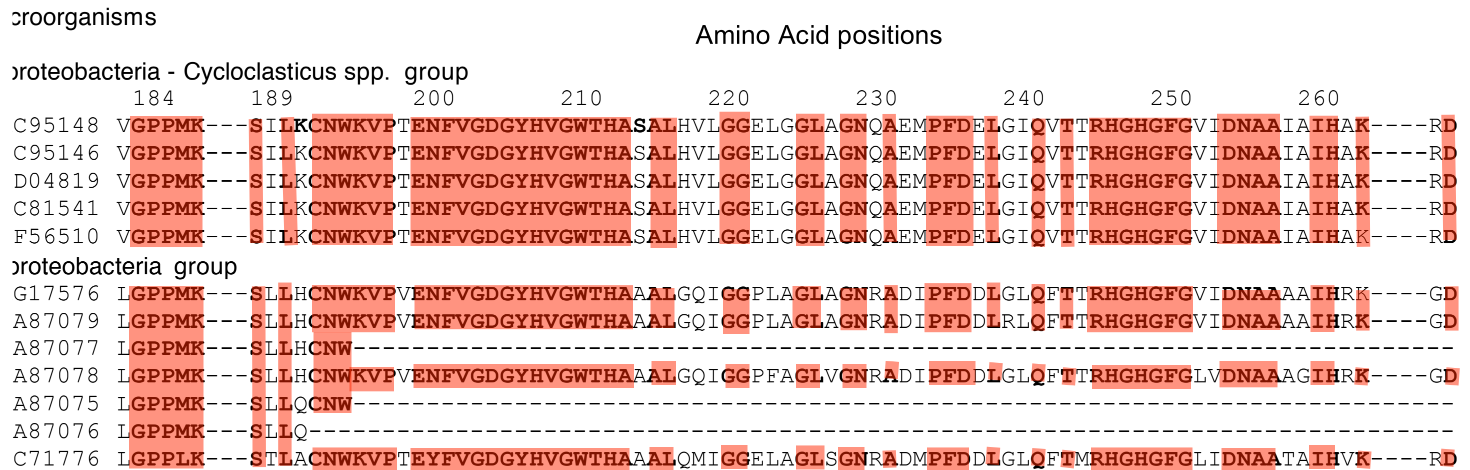

Figure 4.5: Amino acid alignment of the α subunit of PAH dioxygenases from *Cycloclasticus* spp. and members from α-*Proteobacteria* group. Amino acid residues that are homologous have been highlighted.

Phylogenetic analysis of the α subunit sequences grouped *Sphingomonas* sp. A4 (BAD34447) most closely with β-*Proteobacteria* groups II, III and γ-*Proteobacteria* groups I, II and *Pseudomonas* spp. group. However there seems to be little sequence homology between *Sphingomonas* sp. A4 (BAD34447) and the species within the γ-*Proteobacteria* – *Pseudomonas* spp. group and β-*Proteobacteria* group II (figure 4.3). *Sphingomonas* sp. A4 (BAD34447) has regions of sequence homology with the species within the β-*Proteobacteria* group III (figure 4.6).

The α subunit-based analysis has clustered a majority of the *Actinobacteria* as a separate group (see *Actinobacteria* group in figure 4.1) which is consistent with 16S rRNA-based analysis. This may indicate little lateral transfer between *Actinobacteria* and *Proteobacteria*. An insertion and deletion within the α subunit sequences was identified as unique to the *Actinobacteria* (figure 4.7). This supports that there is little lateral transfer between the *Actinobacteria* and the *Proteobacteria*.

```
Microorganisms                                       Amino acid positions
β-Proteobacteria group 3     90         100        110     193    200        210                        230   234
AAN74948 IKAFLNVCRHRGARLCA-VEAGNARGFACNYHGWAYGADGSLL ANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQ
AAN74946 IKAFLNVCRHRGARLCA-VEAGNARGFACNYHGWAYGADGSLL ANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQ
AAN74947 IKAFLNVCRHRGARLCA-VEAGNARGFACNYHGWAYGADGSLL ANWKAPSENFVGDAYHVGWTHASALRSGQSGFAGMAGNNVL-PPAGAGLQVTTQ
Sphingomonas sp. A4
BAD34447 VKAFLNVCRHRGARVCP-VEAGNRRAFVCNYHGWSYAADGSLA CNWKAPTENFVGDAYHIGWTHASSLAASRSIFAPMSGNQML-PPAGAGAQIATR

β-Proteobacteria group 3      260    292
AAN74948 HGHGIGALYDVYAGVHDNE RSHLNGTIFPNTSFLTGSG-------
AAN74946 HGHGIGALYDVYAGVHDNE RSHLNGTIFPNTSFLTGSG-------
AAN74947 HGHGIGALYDVYAGVHNNE RSHLNGTIFPNTSFLTGSG-------
Sphingomonas sp. A4
BAD34447 FGHGLGILYDVNPGVHTAQ GSHLNGSLFPNVSYLWGTN-------
```

Figure 4.6: Amino acid alignment of the α subunit of PAH dioxygenases from members from β-*Proteobacteria* group III and *Sphingomonas* sp. A4. Amino acid residues that are homologous are in boldface.

```
Microorganisms
                                                      Amino acid positions
Actinobacteria spp.                                 220           311
Terrabacter sp. HH4                 (AAZ38356) LG------LLP GQSMPMPILTLR
Mycobacterium vanbaalenii PYR-1     (AAY85176) LG------LLP GESMPVPILTLR
Mycobacterium sp. 6PY1              (CAD38643) LG------LAP KKSMPVPMLTFR
Pseudomonas spp.
Pseudomonas sp. PR3MN2              (AAG25694) SGESIFSSLAG SG-------VFK
Pseudomonas sp. 8IDINH              (AAG25693) SGESIFSSLAG SG-------VFK
Pseudomonas putida                  (AAB47591) SGESIFSSLAG SG-------VFK
β-Proteobacteria group 2
Ralstonia sp. NI1                   (BAB84018) SGQSIFTPLAG SG-------VFK
Delftia acidovorans                 (AAQ84687) SGQSIFTPLAG SG-------VFK
Burkholderia sp. SOD-5b             (AAL46988) SGQSIFTPLAG SG-------VFK
γ-Proteobacteria group 1
Pseudoalteromonas sp. EH-2-1        (AAG18429) TGESVFTPLAG SG-------VFK
Neptunomonas naphthovorans          (AAD04817) TGQSVFTPLSG SG-------VFK
Neptunomonas naphthovorans          (AAD04818) TGESVFTPLAG SG-------VFK
β -Proteobacteria group 3
Burkholderia sp. VUN,013            (AAT37559) SGQSGFAGMAG ------------
Ralstonia sp. PJ531                 (BAB84024) SGQSGFAGMAG SG-------VFK
Burkholderia sp. RP007              (AAD09872) SGQSGFAGMAG SG-------VFK
γ-Proteobacteria group 2
Pseudomonas fluorescens             (AAT37557) SGQSCFAGMAG ------------
Stenotrophomonas maltophilia        (AAT37556) SGQSCFAGMAG ------------
Stenotrophomonas maltphilia         (AAT37558) SGQSCFAGMAG ------------
α-Proteobacteria group
Sphingomonas sp. A4                 (BAD34447) ASRSIFAPMSG TN-------TLK
Sphingomonas sp. CHY-1              (CAG17576) QIGGPLAGLAN TN-------TFK
Sphingomonas sp. HL1                (ABA87079) QIGGPLAGLAN TN-------TFK
γ-Proteobacteria – Cycloclasticus spp. Group
Cycloclasticus sp. W                (AAC95148) VLGGELGGLAG TN-------IFK
Cycloclasticus pugetti              (AAC95146) VLGGELGGLAG TN-------IFK
Cycloclasticus sp. P-1P32           (AAD04819) VLGGELGGLAG TN-------IFK
β -Proteobacteria group 1
Burkholderia sp. Cs1-4              (AAQ84685) ALGGELAAIGG TN-------TFK
Burkholderia sp. Ch3-5              (AAQ84684) ALGGELAAIGG TN-------TFK
Burkholderia sp. Ch1-1              (AAQ84683) ALGGELAAIGG TN-------TFK
```

Figure 4.7: Sequence alignments showing indels within the α subunit of PAH dioxygenases among representatives of PAH-degrading species from major bacterial divisions. Note that the *Actinobacteria* members carry an insertion and deletion.

Discussion

In this study the α subunit of PAH dioxygenases from previously reported PAH-degrading bacterial species have been phylogenetically analysed, in order to clarify the spread of PAH dioxygenases between bacterial species. Determining the extent to which this gene has spread across the divisions of bacteria reveals the ability of adaptation of microbial communities to environmental pollutants. This understanding affords the ability to assess potential species for use within bioaugmentation and the development of bioremediation techniques.

Phylogenetic analysis of the α subunit fragment has resulted in a phylogeny that contain discrepancies in the tree topology when compared to the 16S rRNA-based phylogeny particularly within the α-, β- and γ-*Proteobacteria.* Additionally there is inconsistency within the *Actinobacteria*, which may indicate lateral transfer of the α subunit between the *Actinobacteria* species. Two major lineages were identified from the α subunit-based analysis: *Actinobacteria* and *Proteobacteria*. A majority of the *Actinobacteria* have formed a single group, which is consistent with the 16S rRNA-based analysis (see figure 4.1). This may indicate that there is little lateral transfer between the *Actinobacteria* and *Proteobacteria.* Additional evidence for little lateral transfer between *Actinobacteria* and *Proteobacteria* arises from the identification of indels that are unique to the *Actinobacteria* (see figure 4.7).

There is inconsistency in the tree topologies of the α subunit- and 16S rRNA-based analyses within the *Proteobacteria*. The α-, β and γ-*Proteobacteria* do not form separate monophyletic groups in the α subunit-based phylogeny, which is inconsistent with the 16S rRNA-based phylogeny (figure 4.1 and 4.2). Discrepancies of particular interest arise where taxonomically diverse species from different classes of

Proteobacteria have clustered together: γ-*Proteobacteria-Pseudomonas* spp. group and β-*Proteobacteria* group II; γ-*Proteobacteria* group II and β-*Proteobacteria* group III; γ-*Proteobacteria-Cycloclasticus* spp. group and α-*Proteobacteria* group. Additionally, the presence of highly conserved regions of the α subunit fragment that are shared by taxonomically diverse species support that the α subunit fragment has been laterally transferred between different classes within the *Proteobacteria*. The α subunit genes analysed in this research have been isolated from geographically distinct locations and therefore it would be difficult to conclude that lateral transfer has occurred without accepting long-distance dispersal mechanisms for genes and/or microorganisms. A similar result has been observed by Herrick *et al* (1997) and Matheson *et al* (1996) where they found high sequence homology of a particular gene isolated from geographically disparate locations. These findings may be explained by the Baas-Becking hypothesis that microbes have a ubiquitous nature and can proliferate within any preferred environment (Baas-Beckling, 1984).

The α subunit phylogeny has clustered the γ-*Proteobacteria-Pseudomonas* spp. group more closely to β-*Proteobacteria* group III than to the other γ-*Proteobacteria* groups. Similarly γ-*Proteobacteria* group II has clustered more closely to β-*Proteobacteria* group III than to the other γ-*Proteobacteria* groups. The γ-*Proteobacteria-Cycloclasticus* spp. group is the most distantly related to the other γ-*Proteobacteria* groups and has clustered closest to α-*Proteobacteria* group. This may indicate that lateral transfer of the α subunit gene has occurred after separation of the classes of *Proteobacteria* and after the separation of the genera of the γ-*Proteobacteria*.

The α subunit phylogeny shows that some groupings consist of a single species (e.g. *Pseudomonas* spp. or *Cycloclasticus* spp.) whilst other species (e.g. *Burkholderia* spp.) have formed multiple separate groupings. It has been shown that catabolic genes may undergo intracellular rearrangement and the α subunit has been located both chromosomally and within plasmids (Herrick, 1997). This would account for the high sequence homology across the *Pseudomonas* spp. and *Cycloclasticus* spp. It would also account for lateral transfer of different homologs of the α subunit gene to *Burkholderia* spp. resulting in multiple groups.

The findings of this research indicate the extent of lateral transfer of the α subunit gene, which can be transferred between classes of *Proteobacteria* and between species belonging to the *Actinobacteria*, however, not between the *Actinobacteria* and the *Proteobacteria.* The presence of two major lineages of the α subunit of PAH dioxygenases has been previously noted (Pieper *et al*, 2004). It was determined that the dioxygenases involved in PAH degradation by Gram-positive organisms are only distantly related to dioxygenases of Gram-negative strains (Pieper *et al*, 2004). The presence of PAH-degradative capabilities within the *Actinobacteria* and *Proteobacteria*, yet lack of sequence homology may indicate that the dioxygenase genes evolved prior to the separation of *Actinobacteria* and *Proteobacteria* and that divergent evolution has led to the variation now observed. Conformity to most of the proposed amino acid residues of importance for functionality by the *Actinobacteria* and *Proteobacteria* (Parales *et al,* 2000; see table 4.3) supports that the α subunit of PAH dioxygenases evolved early and natural selection has maintained its function while divergent evolution has resulted in a lack of homology of the α subunit fragment from the *Actinobacteria* and the *Proteobacteria*.

The findings of this research could be explained through the assumption that the α subunit fragment evolved early, prior to the separation of the *Actinobacteria* and *Proteobacteria,* and has undergone divergent evolution to result in different homologs. This would account for the lack of homology of the α subunit from *Actinobacteria* and *Proteobacteria.* The α subunit fragments of *Proteobacteria* have then been laterally transferred between the classes of *Proteobacteria* at different points in time. Additionally there is evidence of the lateral transfer of the α subunit fragment between different *Actinobacteria* species. Early evolution of the α subunit prior to the separation of the *Actinobacteria* and *Proteobacteria* would account for the lack of homology of the α subunit from *Actinobacteria* and *Proteobacteria*, however also accounts for the evidence of lateral transfer of the α subunit (Herrick *et al,* 1997; Stuart-Keil *et al*, 1998; Wilson *et al*, 2003).

These findings have practical implications when probing a microbial population to assess PAH-degradative potential; multiple α subunits need to be targeted in order to successfully identify α subunits of species from both *Actinobacteria* and *Proteobacteria*. It may even be necessary to design genus-specific primers. Furthermore, this study has revealed that lateral transfer of the α subunit occurs extensively between the classes of *Proteobacteria* and therefore utilisation of these species for bioaugmentation techniques may result in successful bioremediation of PAH-contaminated sites. Understanding the extent and frequency of gene transfer between microorganisms may further our understanding of the evolution of microbial populations and subsequently result in improved strategies for manipulating these populations to enhance bioremediation of pollutants *in situ*.

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CHAPTER FIVE

General discussion

5 General discussion

5.1 Introduction

For thousands of years humans have exploited microbial populations within many different processes, for example alcohol production, bread making and more recently wastewater treatment, bioremediation, oil recovery and the production of various chemicals and pharmaceutical compounds through fermentation. This study has focused on the degradative capacity possessed by microbes towards hydrocarbons and other compounds within oil. This degradative capacity can be utilized by humans to remediate polycyclic aromatic compound (PAC)-contaminated sites.

PACs are widespread in nature due to both their natural production in the environment and input from anthropogenic activities, such as the burning of fossil fuels, the use of wood preservatives such as creosote and the generation of wastes from coal gasification plants (Ni Chadhain *et al*, 2006). Some PACs, especially the higher molecular weight compounds, are toxic to living organisms and this toxicity is enhanced by their intrinsic chemical stability and resistance to many forms of degradation (Boffetta *et* al 1997). Therefore, there is now great interest in developing strategies to remove PACs from contaminated sites. 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). For these applications it is important to know more about the diversity of microorganisms that are able to degrade PACs and

the functional catabolic genes involved.

Anthracene (ANT), phenanthrene (PHE) and dibenzothiophene (DBT) have been highlighted as having carcinogenic and mutagenic properties together with a near ubiquitous distribution in depositional environments (Yang *et al*, 1998). Polycyclic aromatic compounds (PACs) such as those mentioned above, pose great environmental concern and therefore were investigated to development strategies to remove PACs from contaminated sites. Determining the microbes within PAC-degradative population together with the associated functional genes will help us towards a better understanding of the biodegradation of these compounds, contributing to the development of more effective bioremediation procedures.

5.2 The impact of the use of a carrier solvent on the enrichment of hydrocarbon-degrading bacterial communities

This study determined the effect that acetone as a carrier solvent has on the process of isolating PAH-degrading bacteria from a sediment sample. Significant shifts in dominant bacterial species in the presence/absence of acetone were observed, which is evidence that the use of a carrier solvent may significantly affect the resultant bacterial population and as such may not represent the degradative community that was originally selected for. The use of a carrier solvent within enrichment studies where the substrate is not water soluble is a common occurrence (e.g. Izumi *et al*, 1994; Moody *et al*, 2001; Nadalig *et al*, 2002). Accordingly, the reliability of the identification of degradative

species that have been isolated in the presence of a carrier solvent is questionable in light of the results of this research.

Enrichment with PAH crystals ensures that the resultant microbial population has been selected on the basis that it can grow on PAHs as the sole carbon source and therefore represent PAH-degrading bacteria. This experimental approach has been adopted in numerous studies (e.g. Ahn *et al*, 1999; Andreoni *et al*, 2004; Ni Chadhain *et al*, 2006). These studies and the findings of this research are evidence that carrier solvents are not always necessary and should be avoided to prevent misleading results.

Other studies have clearly demonstrated that the form in which the PAH substrate is available to the microbes can also significantly affect the composition of the resultant degradative population (Bastiens *et al*, 2000; Cavalca *et al*, 2008; Guerin and Boyd, 1997; Johnsen *et al*, 2005). PAHs in the environment frequently sorb to organic matter, therefore, for the purpose of enriching a bioremedial microbial population it may be important to utilise an experimental design where the PAHs are supplied sorbed to a solid phase. This would allow for enrichment of hydrophobic and adhering PAH-degrading bacteria (Bastiens *et al*, 2000). Such an approach would select for a microbial community that contained the physiological properties that would enhance the bioavailability of PAHs. However, non-aqueous phase liquids (NAPLs) in the environment further complicate PAH bioavailability (Cavalca *et al*, 2008). The complex and heterogeneous nature of *in situ* conditions mean that enriching for a competent degradative community that can be applied to PAH-contaminated sites would be difficult. Careful deliberation is

required when establishing an enrichment culture to ensure the resultant population will be effective in its defined purpose e.g. bioremediation, bioaugmentation.

5.3 Culture-dependent and culture-independent techniques for investigating PAH degradation

If a competent PAH-degradative community is required for use within future bioremediation strategies, then a culture-dependent technique is obviously required. However, if it is necessary to characterise PAH-degradative natural microbial communities, then culture-independent techniques may be more appropriate. It is well known that the majority of microbes in environmental samples (90-99%) cannot be cultured at present on laboratory media, which is biased for the growth of specific microorganisms (Zocca *et al*, 2004). Bakerman and Madsen (2002) investigated the diversity of 16S rRNA and naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters using culture-based techniques and molecular approaches. As expected, clear contrasts between culture-based and nonculture-based α subunit sequences from naphthalene dioxygenases (*nahAc*) were found. Growth-based pure culture techniques revealed only *Pseudomonas*-type bacteria and *nahAc* alleles in the contaminated areas, while molecular techniques detected apparent additional naphthalene-degrader diversity as indicated by *nahAc* sequence polymorphisms (Bakermans and Madsen, 2002). *Pseudomonas boreopolis* was present in the ANT, PHE and DBT enrichments; this may indicate that this is a key PAH/PAC-degrader, although it is recognized that Pseudomonads grow well under laboratory conditions and this may emphasize the bias introduced when applying culture techniques. In addition, culture-

based methods generally differ in physiological conditions to natural ecosystems, particularly nutrient concentration (Watanabe and Hamamura, 2003). Despite these difficulties, tools are being developed to attempt to more accurately assess microbial abundance and distribution in the natural environment and ultimately to link community dynamics with ecosystem functions.

To further the findings of this study, culture-independent techniques could be utilised whereby dominant bacterial species at ANT, PHE and DBT-contaminated sites are determined. The species identified by culture-independent techniques could then be compared to those identified through the enrichments of the current study. If a great distinction exists between the culture-dependent and culture-independent techniques for identifying PAC-degrading species, it may be necessary for future bioremediation strategies to utilise PAC-degrading communities that have been established in the environment to be transferred to other locations e.g. bioaugmentation.

Analysis of DNA extracts from environmental samples is an effective way to account for a very large proportion of microbes in the environment. DGGE allows us to directly determine the presence and relative levels of different 16S rRNA gene amplicons both qualitatively and semiquantitatively in order to perform a community analysis. However, in 16S rRNA gene diversity studies of contaminated sites the functions of the retrieved communities remain unknown, whereas in functional marker gene studies, a function is directly connected to the retrieved community (Sipila *et al*, 2006). More information is available when targeting specific isolates, genotypes or metabolic activities via gene

probes. This approach allows direct identification of the functional active members of the bacterial community which can be done quantitatively via quantitative probing, quantitative PCR and real-time PCR. It is also possible to analyse the *in situ* activity, to determine if environmental clones of catabolic genes are actually expressed *in situ* via reverse transcriptase PCR (Watanabe and Hamamura, 2003). It can be difficult to link the community diversity determined by 16S rRNA gene analysis with the results obtained through targeting functional genes, which could be alleviated through *in situ* PCR coupled to fluorescence *in situ* hybridization (FISH) or stable isotope probing (SIP, e.g. (Boschker and Middelburg, 2002; Manefield *et al,* 2002).

Molecular techniques, however, are not without limitations or bias. Unlike rRNA gene sequences, only a limited number of sequences for catabolic genes are available for the design of PCR primers and probes. In addition, most of these genes have been cloned from laboratory strains that could be easily cultured and therefore can only detect a limited component of the diverse environmental catabolic gene pool (Watanabe and Hamamura, 2003). Other unavoidable methodological and genetic biases which distort sequences from their original *in situ* abundances in the sampled microbial community include: cell lysis, DNA extraction, primer annealing, multiple PCR cycling and varying gene copy numbers of rRNA (Bakermans and Madsen, 2002).

Degradation of pollutants *in situ* occurs amongst a multitude of interactions between microorganisms. To understand the ecological processes involved, identification of functionally important populations is considered to be the primary step, which can then

be followed by isolation and laboratory pure-culture studies of the important organisms (Watanabe and Hamamura, 2003). Molecular techniques can then be employed to develop a greater understanding of the *in situ* activity and interactions within the degradative population. It may be that these different approaches need to be conducted in coordination with each other to further our understanding of the key pollutant degraders in a context which is applicable in the environment.

5.4 Global distribution and diversity of PAC-degrading bacteria

This research 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. This study successfully isolated and characterised three distinct stable PAC-degrading bacterial populations, which may subsequently be utilised in the development of bioremediation techniques.

The findings of this research, together with previous reports (e.g. Andreoni *et al*, 2004; Johnsen *et al*, 2005; Vinas *et al*, 2005, Widada *et al*, 2002) highlight the great diversity of microbial species that can degrade PACs and which therefore could be exploited within bioremediation strategies. PACs are pervasive in the environment; it is therefore advantageous that such a broad diversity of microbial species, each with differing physiological properties, could potentially be applied to the various PAC-contaminated sites. Additionally, a multitude of bacterial species from differing divisions will possess distinct genomic characteristics that could be harboured in bioaugmentation techniques. Characterisation of key PAC-degrading bacteria permits the identification of 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.

Separate enrichments of a single sediment sample with three PAC substrates resulted in three distinct degradative microbial communities. Such a finding reveals the necessity, when sourcing strains to remediate sites contaminated with multiple PACs, to utilise a complex microbial community that encompasses many different genera from the α-, β-, γ-*Proteobacteria* and *Actinobacteria*.

Comparison of the PAC-degrading species enriched from a single sediment sample in our study with previously published research revealed the global distribution of PAC-degrading bacteria as species 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. This finding 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, 1984). Mueller *et al* (1997) similarly concluded that PAH degradation capabilities were associated with members of certain taxa, independent of the origin of the soils from which the bacteria were isolated, which indicates that such taxa are ubiquitously

distributed. Additionally, pristine soils have been found to contain PHE-degrading species, despite there being no previous exposure to PAHs (Johnsen and Karlson, 2005). This further supports the suggestion that PAC-degrading bacteria have a global distribution, which when enriched with a PAH substrate are able to exploit this carbon source.

The idea of Baas-Becking was that microbial life is distributed worldwide and can adapt to any given environmental conditions (de Wit and Bouvier, 2006). However, it is important to note that many studies assign microbes to different species if their ribosomal DNA is less than 97% identical (Konstantinidis and Tiedje, 2005), which may be too low to accurately separate bacterial species and therefore falsely imply a cosmopolitan existence for all microbes (Whitfield, 2005). It has been suggested that this value should be changed to 99% (Stackebrandt and Ebers, 2006). Significantly, if a more stringent similarity level is taken (0.99) then the sequencing analysis of the clones analysed in this study still show support for the Baas-Beckling hypothesis. One ANT clone had 99% sequence homology to a bacterium isolated from rice field soil in Italy (Graff *et al*, 2003) and a DBT clone had 99% sequence homology to an uncultured bacterium clone isolated from urban aerosols from Texas (Brodie *et al*, 2007).

This study supports the idea that microbes will proliferate in an environment containing an energy source that may be utilized and therefore supports Baas-Becking's "everything is everywhere, the environment selects" hypothesis. It appears that the ability to adapt to the environment is great in microorganisms through their ability to transfer functional

genes, high densities and shorter generation times which allow them to undergo rapid genetic divergence (Hughes Martiny *et al*, 2006). Lateral transfer of PAC degradation genes have been previously researched (Herrick *at al* 1997; Johnsen *et al*, 2005; Wilson *et al*, 2003) and so provides a possible explanation for the global distribution of PAC-degradative capabilities.

5.5 Multiple lateral transfer events of PAH dioxygenase gene between different classes of *Proteobacteria,* but limited between *Actinobacteria* and *Proteobacteria*

Understanding how bacteria acclimatise to environmental pollutants is vital for exploiting these mechanisms in clear-up strategies for contaminated sites. To investigate the evolution of PAH-dioxygenases, phylogenetic methods were used to determine the congruence of tree topology for the α subunit of PAH-dioxygenases with a 16S rRNA sequenced-based phylogeny, which is generally accepted as representing the overall phylogeny of the species (Anzai *et al*, 2000; Herrick *et al*, 1997). This study determined the extent to which the PAH dioxygenase gene may have been laterally transferred between the classically recognised divisions of bacteria: multiple lateral transfer events of the α subunit gene were inferred between different classes of the *Proteobacteria*. Similarly, lateral transfer of the α subunit gene appears to have occurred between species of the *Actinobacteria*. However, this study detected no evidence of lateral transfer of the α subunit gene between the *Proteobacteria* and the *Actinobacteria*. Two major lineages within the α subunit phylogeny were formed, indicating distinct α subunit alleles of the PAH dioxygenases.

Two major lineages were identified from the α subunit of PAH dioxygenases phylogeny, which correspond to the taxonomic groups *Actinobacteria* and *Proteobacteria* (as defined by 16S rRNA sequence analysis). There seemed to be no evidence of lateral transfer of the α subunit gene between these two major divisions of bacteria, a finding further supported by the presence of indels unique to the *Actinobacteria*. It has been previously highlighted that the dioxygenases involved in PAH degradation by Gram-positive organisms are only distantly related to the PAH dioxygenases of Gram-negative strains (Pieper *et al*, 2004). The distinction between the α subunit sequences of the *Proteobacteria* and the *Actinobacteria* is consistent with the phylogeny of the species, which indicates that these phyla are distantly related. These findings give rise to the question as to whether the α subunit of PAH dioxygenase evolved separately in the *Actinobacteria* and *Proteobacteria* or whether PAH degradation evolved prior to the separation of these phyla and subsequent divergent evolution resulted in the homologs seen today.

Most of the α subunit amino acid sequences from both the *Actinobacteria* and *Proteobacteria* contain the amino acid residues that have been identified as being important for proper functioning of the dioxygenase. The homology of these residues in both the *Actinobacteria* and the *Proteobacteria* suggests that the α subunit evolved prior to the separation of these phyla and their importance to the proper functioning of the dioxygenase has ensured that natural selection maintained these residues. Additionally, the homology of these residues within the α subunit alleles from the *Actinobacteria* and the *Proteobacteria* indicates the same enzymatic function of the α subunit as part of PAH

dioxygenases; this finding provides further support for the suggestion that the dioxygenase evolved prior to the separation of these phyla.

The ubiquitous nature of the PAH-degradative genes in the environment, at pristine as well as contaminated sites (Laurie and Lloyd-Jones, 2000; Lloyd-Jones *et al*, 1999) provides additional support for the evolution of an ancestral aromatic dioxygenase that has undergone divergent evolution. It appears that the presence of PAH dioxygenases are not limited to PAH-contaminated environments and therefore their existence is not a response to increasing anthropogenic pollution, rather it is a constitutional function that was subsequently adapted to the breakdown of PAHs to exploit the increasing prevalence of PAHs in the environment.

Wilson *et al* (2003) identified two homologs of NDO; the first was initially isolated from *Pseudomonas putida* NCIB 9816-4 (*nahAc*) and the second was initially isolated from *Burkholderia* sp. strain RP007 (*phnAc*). These may represent major α subunit alleles for the γ- and β-*Proteobacteria* respectively. Lateral transfer of both homologs have been determined *in situ* (Herrick *et al*, 1997; Wilson *et al*, 2003) with each allele becoming dominant in two separate PAH-contaminated sites. The explanations for such an occurrence were: inhibition of one phenotype; the mixture of PAHs being more susceptible to attack by one phenotype; lateral transfer of one genotype occurring to a greater extent; one genotype allowed more rapid growth in a particular environment (Wilson *et al*, 2003). However, each allele seems associated with a particular genus and therefore the dominance of an allele may have been coincidental with the proliferation of

the genus most appropriate to the ecological niche. Lateral transfer of *nahAc* and *phnAc* seemed to only occur within the *Pseudomonas* spp. and *Burkholderia* spp. (respectively), which contrasts with the findings of this study which reveals that lateral transfer events have occurred between these genera.

Although there was no evidence for lateral transfer of the α subunit between the *Actinobacteria* and *Proteobacteria*, it is worth noting that other research has identified the polar transfer of antibiotic resistance genes from Gram-positive cocci to Gram-negative bacteria (Courvalin *et al*, 1994). Therefore it is possible PAH degradative capabilities may have evolved originally in the *Actinobacteria*, which was then laterally transferred to the *Proteobacteria*. Such lateral transfer would have been in the distant past as comparison of the α subunit and 16S rRNA phylogenies of this study show no evidence of lateral transfer between these phyla.

In this study, comparison of the α subunit phylogeny with the 16S rRNA phylogeny indicates that there were multiple lateral transfer events of the α subunit between the classes of the *Proteobacteria*. This is further supported by the presence of highly conserved regions of the α subunit fragment within taxonomically diverse species. This reveals the extent to which the α subunit could be promiscuously transferred across the classes of the *Proteobacteria* and would allow microorganisms of this genus to adapt to PAH pollutants within the environment.

Clustering of the α subunit sequences from γ-*Proteobacteria* has revealed that lateral transfer of the α subunit gene has occurred after separation of the classes of *Proteobacteria* and after the speciation of γ-*Proteobacteria*. If the α subunit had evolved in congruence with the overall evolution of the species then one would expect the α subunit phylogeny to appear as in figure 5.1. In this case, the α-, β- and γ-*Proteobacteria* form monophyletic groups and the α subunit sequences of each class are most similar to others in the same class. Also, the α subunit sequences from the genera of the γ-*Proteobacteria* (e.g. *Pseudomonas* and *Cycloclasticus*) would have higher homology than do either of these groups with the α- or β-*Proteobacteria*.

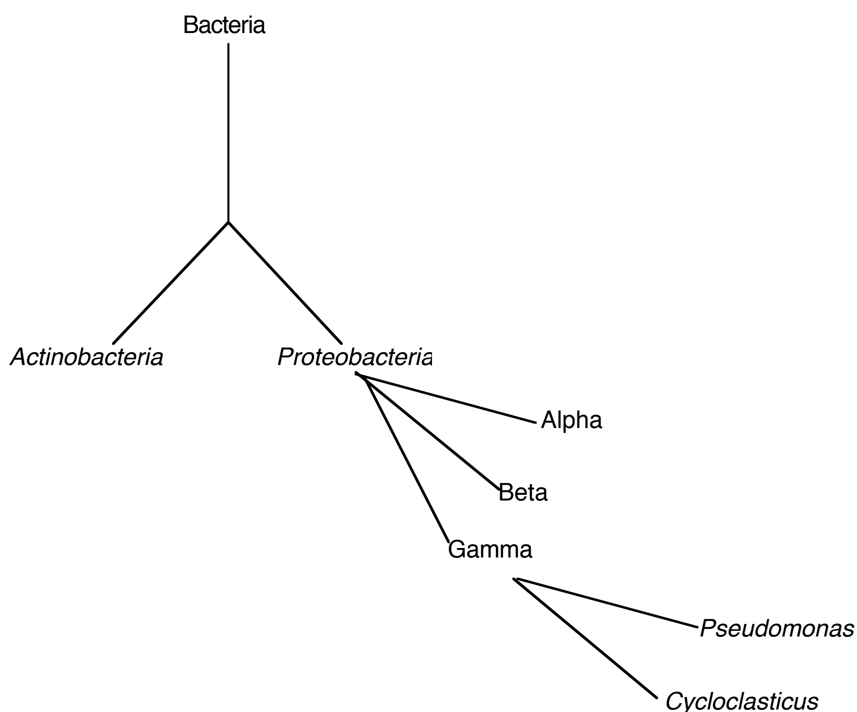

Figure 5.1 Expected phylogeny of the α subunit of PAH dioxygenase if the gene had evolved with the evolution of the species.

Alternatively, if the α subunit evolved prior to the separation of the classes of *Proteobacteria* and then natural selection acted to maintain the sequences within all of the classes there would be little or no difference between the α subunit sequences and we would not see separate clusters forming, as was the case within the phylogenetic analysis undertaken in this study.

It appears that the α subunit has been laterally transferred after the separation of the classes of *Proteobacteria*, as the α subunit from some of the β- and γ-*Proteobacteria* have greater sequence homology with different classes than to sequences isolated from within the same class. In the case of the γ-*Proteobacteria*, one group (*Cycloclasticus* spp. group) is quite distantly related to the other γ-*Proteobacteria* groups, which have significantly greater sequence homology with α subunit genes from β-*Proteobacteria*. It appears that the α subunit has been laterally transferred after the speciation of the γ-*Proteobacteria*, as the α subunit sequences isolated from different genera of the γ-*Proteobacteria* have higher sequence homology with sequences isolated from α- or β-*Proteobacteria*. Accordingly, the α subunit phylogeny appears more comparable to the schematic below (figure 5.2) whereby β- and γ-*Proteobacteria* have formed multiple clusters and genera from distinct classes have clustered closely. This indicates that lateral transfer of the α subunit has occurred between the classes of the *Proteobacteria* after the separation of the α-, β- and γ-*Proteobacteria* divisions. Additionally, lateral transfer appears to have occurred after the speciation of the γ-*Proteobacteria*.

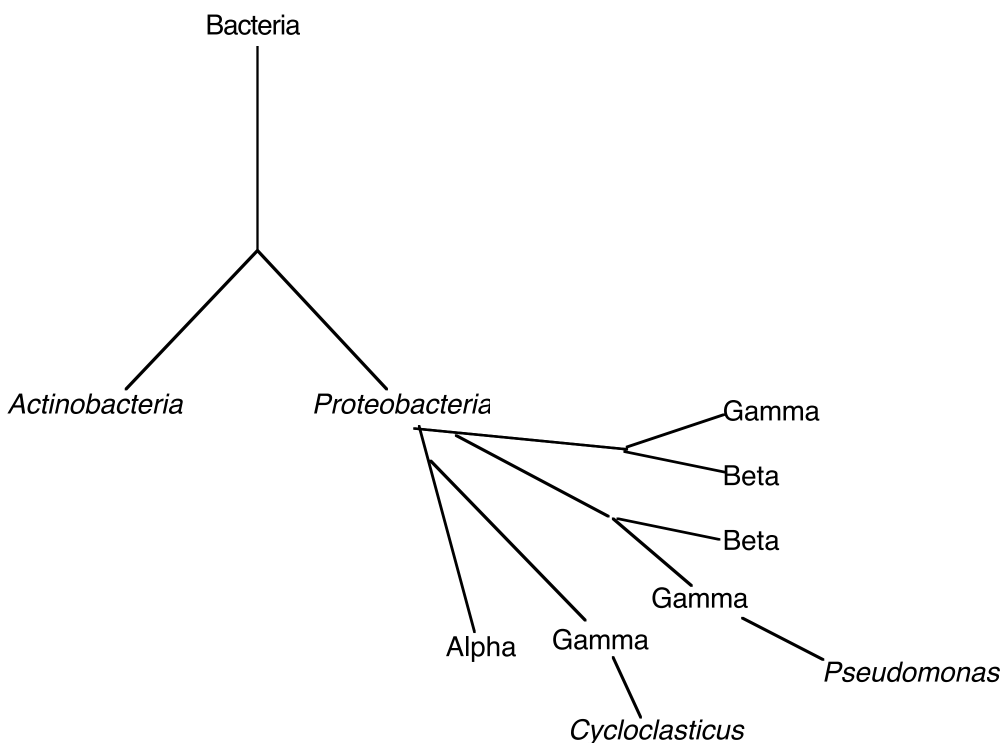

Figure 5.2 Schematic of the α subunit phylogeny as determined in this study.

The α subunit phylogeny contains clusters of highly conserved sequences (e.g. *Pseudomonas* spp. or *Cycloclasticus* spp.) whilst other species (e.g. *Burkholderia* spp.) have formed multiple separate groupings. The occurrence of highly conserved α subunit sequences within genera has been similarly identified within *Pseudomonas*, *Comamonas* and *Rhodococcus* spp. (Moser and Stahl, 2001). It has been shown that catabolic genes may undergo intracellular rearrangement and the α subunit has been located both chromosomally and within plasmids (Herrick, 1997). This would account for the high sequence homology across the *Pseudomonas* spp. and *Cycloclasticus* spp. were vertical transmission may have occurred. It would also account for lateral transfer of different homologs of the α subunit gene to *Burkholderia* spp. resulting in multiple groups.

Within the α subunit phylogeny there are taxa that have not clustered within defined groups. This may be due to the reliability of the sequence data retrieved from GenBank. Alternatively, it may be that these α subunits form part of PAH dioxygenases whose substrate specificity are distinct from the majority of α subunits analysed as it has been established that the α subunit determines the substrate-specificity of the dioxygenase (Parales *et al*, 2000). Such a distinction in the substrate-specificity of these homologs may be due to distinct α subunit sequences and would therefore result in the taxa not clustering within the defined groups.

These findings have practical implications when performing genetic screening of microbial population to assess PAH-degradative potential. A suite of α subunits will need to be targeted in order to successfully identify α subunits of species from both *Actinobacteria* and *Proteobacteria*. It may even be necessary to design genus-specific primers. Due to lateral transfer of the α subunit of PAH dioxygenases between taxonomically diverse species, it may be difficult to infer the taxonomy of the host cell and therefore to utilise such information to establish a defined degradative microbial population. This study has revealed that lateral transfer of the α subunit occurs extensively between the classes of *Proteobacteria*, particularly the β- and γ-*Proteobacteria*, therefore utilisation of these species for bioaugmentation techniques may result in successful bioremediation of PAH-contaminated sites. Understanding the extent and frequency of gene transfer between microorganisms may further our understanding of the evolution of microbial populations and subsequently result in improved strategies for manipulating these populations to enhance bioremediation of pollutants *in situ*.

The development of primer sets which can target a wide range of PAH degradative gene homologs from taxonomically diverse bacterial species is vital for determining the PAH-degradative potential of a given bacterial population. Wilson *et al* (1999) utilized a series of degenerate PCR primers designed to amplify *nahAc* homologs and found that several primer pairs amplified *nahAc* homologs representing the entire diversity of naphthalene-degrading genes (Wilson *et al*, 1999). However, it is also important to identify non-*nah*-like genes. Ni Chadhain *et al* (2006) designed generic PCR primers targeting the gene fragment encoding the Rieske iron sulfur centre common to all PAH dioxygenase enzymes. Many of the Rieske gene fragment sequences fell into clades which are distinct from the reference dioxygenase gene sequences used to design the PCR primers. The ability to profile not only the bacterial community, but also the dioxygenases which they encode, provides a powerful tool for both assessing bioremediation potential in the environment and for the discovery of novel dioxygenase genes (Ni Chadhain *et al*, 2006). Development of a repertoire of PCR primers, including generic primers, would be advantageous for determining the degradative potential of a microbial community. These primers could be applied to the environmental samples and enrichment cultures of this research to determine the α subunit genes present and therefore the catabolically active component of the degradative community.

5.6 Conclusion

Developing a greater understanding of the degradation of PACs/PAHs *in situ* will provide a basis for manipulating bacterial populations for anthropogenic purposes, such as

bioremediation. Various studies have investigated the possibility of bioaugmentation of PAH-polluted soils with PAH-degrading consortia or pure strains, and enhanced remediation of soil slurries in soil-microcosms have indeed been observed. However, to date, there is little evidence of enhanced PAH-biodegradation in large scale experiments as a result of inoculation with PAH-degrading lab-strains (Johnsen *et al*, 2005). It is evident from the findings of this research that traditional enrichment techniques that utilise a carrier solvent significantly affect the rate of PAC degradation and the resultant isolated microbial population. Additionally, previously isolated PAC-degrading species may be unreliable due to the use of a carrier solvent during enrichment. It is clear from published research that other physiological factors may affect the diversity of the enriched bacteria and therefore the use of culture-dependent techniques alone is questionable in the endeavor to isolate a PAC-degradative community for bioaugmentation strategies. Bioaugmentation will require more than simply the addition of a specific catabolic function to contaminated sites. Bioaugmentation may influence the bioavailability of pollutants when the application methods involve homogenization, slurrying or intensive flushing of the system, or when the bacteria added differ from the indigenous population with respect to their specific affinity for the contaminant, maintenance requirements, ability to co-utilise natural substrates, adhesion behaviour, or ability to produce biosurfactants and to ingest surfactant-solubilized chemicals (Johnsen *et al*, 2005). Careful deliberation is required when sourcing strains for bioaugmentation strategies and the use of both culture-dependent and independent techniques may be required for successful clear-up of contaminated sites.

Despite the limitations of using solely culture-dependent techniques for isolating a PAC-degradative population that may be successfully applied to contaminated sites, the PAC-enrichments of this study have revealed the global distribution and diversity of bacterial species that can degrade PACs. Knowledge of the diversity of PAC-degrading bacteria is advantageous to the development of bioremediation as it reveals the multitude of bacterial species, each with individual physiological properties and genetic backgrounds, that can be exploited within tailor-made bioaugmentation strategies. Whether there is successful isolation of a PAC-degradative community *in vitro* or *in situ*, it is encouraging that a great diversity of microbes, which are globally distributed, are able to adapt to PAC pollutants. Understanding the bacteria responsible for these processes is vital for the future harbouring of PAC-degradative capabilities.

To deepen our understanding of PAC-degradation, we should identify not only the microbes responsible but also the functional genes. PAC-enrichments within this study identified the diversity of bacterial species that can degrade PAC and also the ubiquitous nature of these degraders; phylogenetic analysis of a key enzyme, the α subunit of PAH dioxygenases, revealed how PAH-degradative capabilities have become ubiquitous across the divisions of bacteria.

Two major lineages of the α subunit of PAH dioxygenases were identified: the *Proteobacteria* and the *Actinobacteria*. Homology of amino acid residues, identified as important to the functioning of the α subunit, within the sequences isolated from both *Actinobacteria* and *Proteobacteria* imply that the PAH-dioxygenases evolved prior to the

separation of these phyla or that lateral transfer occurred in the very distant past. No evidence for the lateral transfer of the α subunit between the *Actinobacteria* and the *Proteobacteria* was presence in the phylogenetic analyses of this research. Multiple lateral transfer events were inferred within the species of the *Actinobacteria* and between the classes of the *Proteobacteria*. The clustering of the taxa within the α subunit phylogeny indicates that lateral transfer of the gene occurred after the separation of the classes of *Proteobacteria* and also after the speciation of the γ-*Proteobacteria.* These findings reveal how bacteria have acclimatised to PAH pollutants through multiple lateral transfer events of a key PAH-degradative gene. Although lateral transfer may pose some practical problems e.g. primer design, understanding the extent and frequency to which this gene can be laterally transferred will aid further development of bioaugmentation techniques. In addition, the occurrence of lateral transfer of PAH-degradative genes within classes of *Proteobacteria* and species of *Actinobacteria,* and noting the limitation between these phyla, may be similar for degradative genes towards other pollutants and indeed other functionally important genes. Ultimately, this knowledge of the transfer of genetic material should serve to broaden our prospects of usefully exploiting microbial populations.

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