Research Article

Isolation and Identification of Pyrene Mineralizing *Mycobacterium* spp. from Contaminated and Uncontaminated Sources

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Mycobacterium isolates obtained from PAH-contaminated and uncontaminated matrices were evaluated for their ability to degrade three-, four- and five-ring PAHs. PAH enrichment studies were prepared using pyrene and inocula obtained from manufacturing gas plant (MGP) soil, uncontaminated agricultural soil, and faeces from *Macropus fuliginosus* (Western Grey Kangaroo). Three pyrene-degrading microorganisms isolated from the corresponding enrichment cultures had broad substrate ranges, however, isolates could be differentiated based on surfactant, phenol, hydrocarbon and PAH utilisation. 16S rRNA analysis identified all three isolates as *Mycobacterium* sp. The *Mycobacterium* spp. could rapidly degrade phenanthrene and pyrene, however, no strain had the capacity to utilise fluorene or benzo[a]pyrene. When pyrene mineralisation experiments were performed, 70–79% of added ¹⁴C was evolved as ¹⁴CO₂ after 10 days. The present study demonstrates that PAH degrading microorganisms may be isolated from a diverse range of environmental matrices. The present study demonstrates that prior exposure to PAHs was not a prerequisite for PAH catabolic activity for two of these *Mycobacterium* isolates.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that have been widely distributed as a result of anthropogenic activities including the combustion of fossil fuels and organic matter, coal liquefaction and gasification processes, oil seepage, and accidental spillage of hydrocarbons [1, 2]. Due to their acute toxicity and/or mutagenic, teratogenic, or carcinogenic properties [3–5], there is toxicological concern about the presence of PAHs in the environment. As a result, the need to develop inexpensive and practical remediation technologies for PAH-contaminated soil is evident.

Over the past 20 years, bioremediation has been promoted as a potential economic strategy for the remediation of PAH-contaminated soil. A wealth of information has accumulated in the scientific literature on bacterial PAH degradation including rate and extent of degradation, metabolic

pathways, molecular mechanisms of PAH degradation, and application of these organisms to bioremediation strategies. A number of papers have reviewed the catabolic diversity of bacterial, fungal, and algal degradation of PAHs [6–8], whilst the review of Juhasz and Naidu [2] focussed on the microbial degradation of benzo[a]pyrene. It is apparent from these reviews and earlier suggestions by Kastner et al. [9], that nocardioform bacteria, in particular *Mycobacteria*, may play a crucial role in the biodegradation of PAHs.

Traditionally, organisms for PAH bioremediation have been sourced from environments contaminated with the target pollutant(s) on the assumption that there is a better likelihood for these organisms to have potential to degrade PAHs. It is thought that the contaminated environment exerts a selective pressure for organisms with the metabolic capability to degrade the target compound as a carbon and energy source and that the likelihood of isolating an organism with degradative capabilities from these sources is

greater than uncontaminated sources [10]. However, PAH-degrading organisms may be isolated from uncontaminated sources as exemplified by the research of Kanaly et al. [11] and Juhasz and Naidu [12].

In this study, PAH enrichment cultures were prepared in an attempt to isolate pyrene-degrading microorganisms from contaminated (manufactured gas plant soil) and uncontaminated (agricultural soil and kangaroo faeces) sources. Isolated microorganisms were identified by sequencing 16S rRNA gene and substrate utilisation patterns characterised using a microtitre plate method. In addition, the rate and extent of pyrene mineralisation by the isolated microorganisms was compared to a known pyrene-degrading *Mycobacterium* sp. (strain 1B) in ^{14}C -pyrene experiments.

2. Materials and Methods

2.1. Media, Stock Solutions, and Growth Conditions. Enrichment and PAH degradation studies utilised a Basal Salts Medium (BSM) supplemented with PAHs [13]. In some cases, BSM was supplemented with yeast extract (0.05 g/L) (BSMY) and when solid media was required, 15 g Bacteriological Agar 1 (oxide) was added prior to autoclaving.

PAH stock solutions were prepared in dimethylformamide (DMF) at the following concentrations: 5 mg/mL, anthracene, benz[a]anthracene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene and fluoranthene; 10 mg/mL, fluorene; 25 mg/mL, phenanthrene and pyrene. BSM was supplemented with individual PAHs to achieve final concentrations of 50 mg/L for anthracene, benz[a]anthracene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene and fluoranthene, 100 mg/L for fluorene and 250 mg/L for phenanthrene and pyrene. Cultures were incubated at 30°C and 150 rev/min in the dark.

2.2. Source of Inocula. A range of PAH-contaminated and uncontaminated materials were obtained as a source for bacterial inocula. Manufactured gas plant (MGP) soil (0–20 cm) was collected from the site of a former manufactured gas plant in Glenelg, South Australia. Uncontaminated agricultural soil (0–20 cm) was collected from the boundary of a field used for the cultivation of wheat and pea straw in Kanmantoo, South Australia. No industrial activity had been undertaken in the near vicinity of the agricultural plot. Table 1 provides an outline of selected soil properties of MGP and uncontaminated agricultural soil. In addition, six pellets (20 g) of fresh kangaroo faeces (Western Grey Kangaroo, *Macropus fuliginosus*) were collected from the soil surface at Upper Sturt, South Australia.

All samples were stored at 4°C until required. In addition to the above inoculum sources, *Mycobacterium* sp. strain 1B, was used for the comparison of PAH degradative ability. The strain was isolated by Dandie et al. [14] and was capable of degrading phenanthrene, fluoranthene, and pyrene as sole sources of carbon and energy.

2.3. Enrichment and Isolation of PAH-Degrading Bacteria. Bacterial inocula were prepared by shaking 20 g (wet weight)

TABLE 1: Soil properties of manufacturing gas plant soil and uncontaminated agricultural soil.

Parameter	Agricultural soil	Manufactured gas plant soil
Nutrients—% (w/w)		
Total Kjeldahl Nitrogen	0.16	0.42
Boron	<0.001	<0.001
Calcium	0.18	0.44
Copper	<0.01	<0.01
Iron	2.2	2.7
Potassium	0.47	0.22
Magnesium	0.38	0.20
Manganese	0.03	0.02
Sodium	0.02	0.02
Phosphorus	0.02	0.02
Sulphur	<0.01	0.41
Zinc	<0.01	0.02
PAHs (mg kg ⁻¹)		
Naphthalene	<0.10	32
Acenaphthylene	<0.10	9.0
Acenaphthene	<0.10	6.3
Fluorene	<0.10	12
Phenanthrene	<0.10	52
Anthracene	<0.10	43
Fluoranthene	<0.10	64
Pyrene	<0.10	120
Benz(a)anthracene	<0.10	110
Chrysene	<0.10	90
Benzo(b)fluoranthene	<0.10	120
Benzo(k)fluoranthene	<0.10	79
Benzo[a]pyrene	<0.10	94
Indeno(1,2,3-CD)pyrene	<0.10	180
Dibenz(a,h)anthracene	<0.10	10
Benzo(g,h,i)perylene	<0.10	70
Total PAH	<0.10	1100

of each inoculum source in 100 mL of Phosphate Buffered Saline (PBS) (g/L: 80 g NaCl; 2 g KCl; 14.4 g Na₂HPO₄; 2.4 g KH₂PO₄) overnight at 30°C and 150 rev/min in the dark. Following shaking, the samples were left to settle for 1 hour then 5 mL of the supernatant was transferred to BSM (45 mL) containing pyrene at a concentration of 250 mg/L. Enrichments were incubated for up to 10 weeks. Following visible growth of inocula, aliquots of enrichment cultures (5 mL) were aseptically transferred to fresh pyrene-containing medium and incubation continued. This enrichment procedure was repeated for three successive transfers.

Following enrichment, culture supernatants (0.1 mL) were plated onto BSM or BSMY agar plates containing pyrene (250 mg/L). Plates were incubated at 30°C and routinely checked for zones of PAH clearing surrounding colonies. Bacterial colonies showing zones of PAH clearing

were transferred to BSMY-pyrene plates until pure cultures were isolated.

2.4. Identification of PAH-Degrading Bacteria–16S rRNA Analysis. Total DNA was extracted from isolates grown on Luria Bertani Agar (g/L: 10 g Tryptone; 5 g Yeast Extract; 10 g NaCl; pH 7.0) using the DNeasy Plant Mini Kit (Qiagen, Calif USA) as per the manufacturer's instructions. 16S ribosomal DNA was amplified (Perking Elmer Thermal Cycler PE 9700) using the following primer sets (Geneworks, Hindmarsh SA):

- (i) fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') [15]
- (ii) rD1 (5'-AAG GAG GTG ATC CAG CC-3') [15]
- (iii) 27f (5'-AGA GTT TGA TCM TGG CTC AC-3')
- (iv) 765r (5'-TAC GGY TAC CTT GTT ACG ACTT-3')

The PCR products obtained were purified using Promega (MD, USA) Wizard PCR Preps DNA Purification System Kit (for fD1 and rD1) or MoBio UltraClean PCR clean up kit (Geneworks) (for 27f and 765r). Purified DNA samples were sequenced by the Flinders University of South Australia/Flinders Medical Centre DNA Sequencing Core Facility (Department of Haematology, FMC, Adelaide). The consensus sequence was entered into the National Centre of Biotechnology Information (NCBI) database using the nucleotide BLAST (Basic Local Alignment Search Tool) and the nearest relatives identified. Phylogenetic trees of bacterial strains were constructed using Phylip Neighbour Joining method on Ribosomal Database Project II (RDP).

2.5. Preparation of Bacterial Inocula for Degradation Experiments. Bacterial inocula for degradation experiments were prepared in BSM containing pyrene (250 mg/L) as the sole source of carbon and energy. Cultures were incubated for 7 to 14 days then biomass was harvested by centrifugation (14,470 ×g for 10 minutes at 4°C). Cell pellets were washed twice in BSM (20 mL), recentrifuged after each wash, then resuspended in BSM (20 mL).

2.6. Characterisation of Substrate Range for PAH-Degrading Bacteria. The ability of PAH-degrading bacteria to grow on a range of substrates was tested using a microtitre plate system. PAH-degrading bacteria (20 µL) were inoculated into 96 well microtitre plates containing BSM (170 µL) and 10 µL of a particular test substrate. Substrates selected included carbohydrates, PAHs, PAH degradation products, surfactants, and organic environmental contaminants. Substrates were added to BSM to achieve a final concentration of 50 mg/L. Wells containing BSM without substrates and R2A media were used as negative and positive controls. All substrates were sterilised prior to use by either filtration (0.22 µm, Millipore) or autoclaving. Microtitre plates were incubated at 30°C for up to 7 days. Growth at the expense of the supplied substrate was determined based on the presence or absence of observed turbidity. In wells where residual substrate obscured bacterial growth or where turbidity was difficult to distinguish, iodonitrotetrazolium chloride (INT; 10 mg/mL)

was added and incubated for a further 60 minutes at 30°C. Development of a red pigmentation indicated microbial activity and was scored positive for substrate utilisation.

2.7. Degradation of PAHs in Liquid Culture. Bacteria isolated from enrichment studies were tested for their ability to degrade a range of PAHs in liquid medium. Replicate serum bottles containing BSMY (9.9 mL) supplemented with individual PAHs (100 mg/L fluorene; 250 mg/L phenanthrene and pyrene; 50 mg/L fluoranthene and benzo[a]pyrene) were inoculated with 100 µL of the cellular biomass resulting in a final cell density of approximately 10⁵ cells/mL.

Controls consisted of inoculated media without PAHs (supplemented with 0.1 mL DMF) and mercuric chloride killed culture with PAH additions. Incubations were performed in triplicate for each set of culture conditions. Bottles were incubated and samples removed for analysis (depending on PAH supplied) after 2, 4, 5, 8, 10, 15, 20, 28, and 50 days. Growth at the expense of PAHs was established by a most probable number technique (five replicate inoculations) using 96-well microtitre plates containing R2A medium. Following incubation (30°C for up to 7 days), growth was scored by observing the presence or absence of turbidity in wells. The viable count was estimated from the results using statistical tables [16].

2.8. Mineralisation of Pyrene in Liquid Culture. Bacterial isolates were assessed for their ability to mineralise [4,5,9,10-¹⁴C] pyrene (Sigma-Aldrich) in duplicate biometer flasks. Unlabelled pyrene was added to BSMY (20 mL) to achieve a final concentration of 250 mg/L. Each flask was also supplemented with 1.0 µCi of [4,5,9,10-¹⁴C] pyrene (58.7 mCi/mmol). Media was inoculated with cellular biomass resulting in a final cell density of approximately 10⁶ cells/mL. Cultures were incubated for 10-days with pyrene mineralisation determined by monitoring the distribution of ¹⁴C in the culture medium, biomass and gaseous phase.

2.9. Extraction and Quantification of PAHs. PAHs were extracted from culture medium using dichloromethane (DCM) (3 mL) and benzo[b]fluorene (100 µL of 1000 µg/mL) according to Juhasz et al. [13]. Extracts (1 mL) were added to amber glass vials (Varian) and stored at -20°C before analysis by gas chromatography equipped with flame ionisation detection (GC-FID).

The PAH concentration in DCM extracts was quantified using a Varian Star CP-3800 GC-FID equipped with an EC-5 ECONO-CAP capillary column (30 m × 0.25 mm, Alltech, Australia). Injector and detector temperatures were maintained at 320°C and 330°C, respectively, while the oven temperature was programmed at 200°C for 1 minute, followed by a linear increase of 10°C per minute to 300°C and held for 10 minutes [14]. The GC-FID quantification procedure used was similar to the USEPA 8100 method for PAH analysis [17]. The external standard method was used for quantification while benzo[b]fluorene was used as a recovery surrogate. Recovery of benzo[b]fluorene during

PAH quantification ranged from 95–100% for liquid culture extractions with replicate analysis of the same sample showing a standard deviation of less than 1%.

2.10. Detection of Radioactivity. $^{14}\text{CO}_2$ from experiments conducted with $[4,5,9,10-^{14}\text{C}]$ pyrene was collected in a 0.1 M NaOH traps (5 mL) contained in the side arm of biometer flasks. At various time points, the NaOH trap was completely removed for analysis and replaced by fresh NaOH. At the completion of the mineralisation experiments, 10 M HCl (0.5 mL) was added to the medium to liberate any residual $^{14}\text{CO}_2$. Duplicate aliquots (1 mL) of NaOH traps were added to ReadySafe liquid Scintillation cocktail (9 mL; Beckman-Coulter, USA) and analysed for radioactivity on a Beckman Scintillation Counter. To obtain a ^{14}C mass balance, the culture fluid was centrifuged ($3,620 \times g$ for 10 minutes) and duplicate aliquots of the culture supernatant (1 mL) were removed and added to ReadySafe scintillation cocktail (9 mL) for analysis of radioactivity. Cell pellets were extracted with DCM (5 mL) and the beta emissions measured. Finally, to determine the amount of ^{14}C incorporated into cellular biomass, cell debris was washed and resuspended in Milli-Q water (5 mL) and duplicate aliquots (1 mL) added to ReadySafe liquid scintillation cocktail (9 mL) for analysis of radioactivity.

2.11. Statistical Analysis. Prior to statistical analysis, data were tested for normality and homogeneity using SPSS v.14 for Windows. Analysis of variance was conducted at $\alpha = 0.05$ to determine the treatment significance for each parameter for each isolate. Treatment means showing significance were separated using Tukey's Multiple Comparisons test at 5% level of significance. Pearson correlations (r_p) (2-tailed) were preformed to describe the relationship between measured microbial abundance and PAH removal.

3. Results

3.1. Isolation of PAH-Degrading Bacteria. Three pyrene-degrading bacteria were isolated from PAH enrichment cultures containing manufactured gas plant soil, uncontaminated agricultural soil, and kangaroo faeces as the inocula. These organisms grew rapidly and produced distinct clearing zones on BSMY-pyrene plates. The ability of these isolates to degrade pyrene was confirmed following inoculation of individual microorganisms into BSMY-pyrene broths and subsequent visual detection in reduction of pyrene and increase in cellular biomass. All three isolates were Gram positive and morphologically similar. Colonies were circular (≤ 2 mm in diameter) with a smooth/mucoid surface and entire edge. Their pigment and opacity ranged from cream to yellow/orange and translucent to opaque.

3.2. Identification of PAH-Degrading Bacteria. Identification of PAH-degrading isolates was performed using 16S rRNA. PCR products were amplified from the uncontaminated agricultural soil isolate using the fD1-rD1 primer set while an alternative primer set (27f-765r) was used for the manufacturing gas plant soil and kangaroo faeces isolates. The

identity of these sequences were determined using BLAST similarity search accessed through NCBI database. Results of this identity search revealed that the sequence obtained from this isolate showed highest similarity to *Mycobacterium* spp. (Table 2). The isolates were given the following strain identifiers based on their enrichment source:

- (i) uncontaminated agricultural soil isolate: *Mycobacterium* sp. Strain KA5
- (ii) manufactured gas plant soil isolate: *Mycobacterium* sp. Strain BS5
- (iii) kangaroo faeces isolate: *Mycobacterium* sp. Strain KF4.

3.3. Substrate Range of PAH-Degrading Bacteria. *Mycobacterium* sp. strains KA5, BS5, and KF4 were all able to utilise a broad range of carbohydrates (Table 3). No differentiation between the three isolates could be made based on carbohydrate utilisation. Similarly, substrate utilisation patterns for potential PAH metabolites were similar for *Mycobacterium* sp. strains KA5, BS5, and KF4. None of the isolates were able to utilise protocatechuic acid, maleic acid cinnamic acid, *d*-pantothenic acid, or phthalic acid. *Mycobacterium* sp. strain BS5 could be differentiated from strains KA5, and KF4 by its inability to grow on the chlorinated aromatic 3-chlorobenzoate. Greater variation between these isolates was observed in substrate utilisation experiments with surfactants, phenols, hydrocarbons, and PAHs supplied as sole carbon and energy sources (Table 4). *Mycobacterium* sp. Strain BS5 could be differentiated from strains KA5 and KF4 by its inability to grow on the surfactant Tergitol NP-10 while *Mycobacterium* sp. Strain KF4 could be differentiated from strain KA5 by its inability to grow on Tween 80. None of the isolates were able to utilise phenols as growth substrates, however, all *Mycobacterium* sp. strains were able to grow on monoaromatics, *n*-alkanes, and diesel. Slight differences in substrate utilisation patterns were observed for PAHs. No isolate was able to grow on naphthalene, acenaphthylene, fluorene and benzo[*a*]pyrene, however, growth was observed on phenanthrene, pyrene, fluorene, benz[*a*]anthracene, and dibenz[*a,h*]anthracene. *Mycobacterium* sp. strain BS5 could be differentiated from strains KA5 and KF4 by its inability to grow on chrysene while strain KA5 could be differentiated from strain KF4 by its inability to grow on anthracene (Table 4).

3.4. PAH Degradation. The PAH catabolic potential of *Mycobacterium* sp. strains KA5, BS5, and KF4 were assessed over a time course period in BSMY supplemented with individual PAHs. In addition, the PAH degradative profile of *Mycobacterium* sp. Strain 1B, a PAH-degrading bacterium isolated by Dandie et al. [14], was also assessed for comparison. During degradation experiments, abiotic removal of PAHs was minimal in uninoculated or mercuric chloride-killed controls with the exception of fluorene cultures where a $53 \pm 7\%$ decrease was observed presumable due to volatilisation. Degradation experiments confirmed the inability of *Mycobacterium* sp. strains KA5, BS5 and KF4

to grow on and degrade fluorene (Table 5) as observed in substrate utilisation experiments.

3.5. Phenanthrene Degradation. All four *Mycobacterium* species were capable of removing significantly greater concentration of phenanthrene from BSMY compared with abiotic loss ($P < .001$) after 14 days incubation. Variation between isolates was observed in the lag period preceding degradation and the rate of degradation. Degradation lag periods were not observed for *Mycobacterium* sp. strains BS5, KA5, and KF4 following inoculation into medium containing phenanthrene (250 mg L^{-1}) (Figure 1(a)). For *Mycobacterium* sp. strains BS5 and KA5, degradation was rapid with >98% of phenanthrene being removed after 4 days, while complete removal of phenanthrene in cultures inoculated with *Mycobacterium* sp. Strain KF4 required 8 days. A four-day lag period before the onset of phenanthrene degradation was observed for *Mycobacterium* sp. Strain 1B after which phenanthrene was rapidly removed, reducing from $237 \pm 17 \text{ mg/L}$ at day 4 to $19 \pm 2 \text{ mg/L}$ at day 11.

Phenanthrene removal corresponded with an increase in microbial numbers in cultures inoculated with *Mycobacterium* sp. strains BS5, KA5 and KF4. Microbial numbers increased by an order of magnitude during phenanthrene degradation (from 1.5×10^5 to 2.5×10^6 cells/mL), however, following the removal of phenanthrene, decreased to initial values. This trend was not shown to be a significant correlation ($P > .05$), however it is important to note that the majority of contaminant removal occurred in the initial stages of experiments, and this infers that correlations at later time points may not reflect the true relationships between these parameters as they relate to degradation and microbial growth. In cultures inoculated with *Mycobacterium* sp. Strain 1B, microbial numbers decreased following inoculation and did not recover until after the degradation lag period. After this period, microbial numbers gradually increased resulting in microbial numbers similar to those at the commencement of degradation experiments (2.5×10^5 cells/mL). For this species a significant negative correlation between microbial abundance and phenanthrene degradation was shown ($r_p = -0.8309$, $P = .04$).

3.6. Fluoranthene Degradation. The greatest variation in PAH degradative capacity was observed in experiments conducted with fluoranthene (50 mg/L) (Figure 1(b)). While all species were able to degrade significantly greater concentration of fluoranthene compared with abiotic loss ($P \leq .001$), interspecies variation in endpoint degradation outcomes was noted. Complete removal of fluoranthene was observed in cultures inoculated with *Mycobacterium* sp. Strain 1B following a 20-day incubation period. The other *Mycobacterium* species had a decreased ability ($P < .001$) to remove fluoranthene from BSMY: over the 20-day incubation period, 60%, 24% and 20% of fluoranthene was removed by *Mycobacterium* sp. strains KA5, BS5, and KF4, respectively.

Fluoranthene removal in all experiments was preceded by an extended degradation lag period. Reductions in fluoranthene concentration were observed after 5–10-days in cultures inoculated with *Mycobacterium* sp. strains

FIGURE 1: Phenanthrene (a), fluoranthene (b), and pyrene (c) degradation by *Mycobacterium* sp. strains 1B (white square), BS5 (black diamond), KA5, (black square), and KF4 (black triangle). The change in PAH concentration in mercuric chloride killed controls is also shown (black circle). Data points represent the mean and standard deviation of three replicates for each time point.

TABLE 2: 16S rRNA identification of PAH-degrading bacterial isolates from enrichment cultures containing manufacturing gas plant soil, uncontaminated agricultural soil, and kangaroo faeces. The top ten aligned sequences between bacterial isolates and members of the BLAST sequence database are shown.

Isolate	Primer set	Aligned sequences from BLAST	% Similarity
Manufacturing gas plant soil (strain BS5)	27f-765r	<i>Mycobacterium</i> sp. PYR GCK	98
		<i>Mycobacterium gilvum</i> isolate VM0583	98
		<i>Mycobacterium gilvum</i> isolate VM0442	98
		<i>Mycobacterium gilvum</i> isolate VM0552	98
		<i>Mycobacterium gilvum</i> isolate VM0504	98
		<i>Mycobacterium gilvum</i> isolate VM0505	98
		<i>Mycobacterium</i> sp. strain BB1	98
		<i>Mycobacterium</i> sp. HE5	98
		<i>Mycobacterium mucogenicum</i> strain ATCC 49649	98
		<i>Mycobacterium mucogenicum</i> strain ATCC 49649	98
Uncontaminated agricultural soil (Strain KA5)	fD1-rD1	<i>Mycobacterium monacense</i> B9-21-178	94
		<i>Mycobacterium</i> sp. KMS	94
		<i>Mycobacterium</i> sp. JLS	94
		<i>Mycobacterium gilvum</i> isolate VM0442	93
		<i>Mycobacterium gilvum</i> isolate VM0552	93
		<i>Mycobacterium gilvum</i> isolate VM0504	93
		<i>Mycobacterium gilvum</i> isolate VM0505	93
		<i>Mycobacterium</i> sp. PYR GCK	93
		<i>Mycobacterium petroleiphilum</i>	93
		<i>Mycobacterium chlorophenolicus</i> (PCP-1)	93
Kangaroo faeces (Strain KF4)	27f-765r	<i>Mycobacterium</i> sp. JLS	95
		<i>Mycobacterium monacense</i> B9-21-178	95
		<i>Mycobacterium vaccae</i> isolate VM0588	95
		<i>Mycobacterium vaccae</i> isolate VM0587	95
		<i>Mycobacterium</i> sp. KMS	95
		<i>Mycobacterium</i> sp. MCS	95
		<i>Mycobacterium doricum</i> strain DSM 44339	94
		<i>Mycobacterium doricum</i>	94
		<i>Mycobacterium duvalii</i>	94
		<i>Mycobacterium duvalii</i> strain CIP 104539	94

1B, BS5, KA5, and KF4. The extended degradation lag periods observed in cultures inoculated with *Mycobacterium* strains indicates that the lack of fluoranthene removal observed over the incubation period may be a product of the slow degradation rates rather than a lack of fluoranthene degradative capacity.

Microbial growth at the expense of fluoranthene was variable in culture inoculated with *Mycobacterium* species. A reduction in fluoranthene concentration coincided with microbial growth in experiments inoculated with *Mycobacterium* sp. strains BS5, KA5, and KF4, denoted by a significant negative relationship for all strains ($r_p = -0.9407$, $P = .005$; $r_p = -0.9892$, $P = .0002$; $r_p = -0.9867$, $P = .0003$, resp.). In contrast, in *Mycobacterium* sp. Strain 1B inoculated experiments, microbial numbers decreased slightly (7.5×10^5 cells/mL to 1.5×10^5 cells/mL; $P > .05$) during the degradation of fluoranthene.

3.7. Pyrene Degradation. All isolates were efficient at degrading pyrene ($P < .0001$). This was not surprising given pyrene was the substrate used for the isolation of these strains. Over a 20-day incubation period, greater than 94% of pyrene was removed from all *Mycobacterium* inoculated BSMY containing 250 mg/L of pyrene (Figure 1(c)). Unlike experiment using fluoranthene, extended degradation lag periods were not observed in pyrene experiments. This may have been a product of the bacterial isolates used in the experiments being grown on pyrene prior to inoculation. Removal of pyrene occurred immediately in experiments inoculated with *Mycobacterium* sp. Strain 1B and KA5 while a short degradation lag period (2 days) was observed in experiments inoculated with *Mycobacterium* sp. strains BS5 and KF4. Removal of pyrene by *Mycobacterium* species resulted in a significant increase in microbial numbers by an order of magnitude ($P < .001$).

TABLE 3: Growth of *Mycobacterium* sp. isolates on various carbohydrates and potential PAH metabolites. Growth was scored visually on the basis of turbidity and ranked accordingly.

Substrate	<i>Mycobacterium</i> sp. strain BS5	<i>Mycobacterium</i> sp. Strain KA5	<i>Mycobacterium</i> sp. strain KF4
Carbohydrates			
Adonitol, Arabinose, Glucose, Lactose, Maltose, Mannitol, Mannose, Raffinose, Rhamnose, Sorbitol, Sucrose	+++ ^a	+++	+++
PAH metabolites			
Succinate	++	++	+++
Pyruvate	++	++	+++
Salicylate	++ ^b	++	+++
Catechol	+ ^c	++	+++
Cinnamic, Maleic, <i>d</i> -Pantothenic, Phthalic Protocatechuic acids	— ^d	—	—
Sodium benzoate	++	+++	+++
<i>p</i> -aminobenzoic acid	++	++	+++

^ahigh turbidity, ^bmedium turbidity, ^clow turbidity, ^dno observed turbidity.

3.8. Pyrene Mineralisation. To confirm pyrene degradation by the *Mycobacterium* species, radiolabelled experiments were conducted using [4,5,9,10-¹⁴C] pyrene. Figures 2(a) and 2(b) present the results from pyrene mineralisation experiments illustrating the evolution of ¹⁴CO₂ over the time course period and a mass balance of ¹⁴C at the conclusion of the experiment. Over the 10-day incubation period, the extent of pyrene mineralisation by the *Mycobacterium* species was similar: 74%, 70% 79%, and 71% of ¹⁴C was recovered as ¹⁴CO₂ in cultures inoculated with *Mycobacterium* sp. strains 1B, BS5, KA5, and KF4, respectively. At the conclusion of the experiment, less than 3% of the ¹⁴C was detected in the culture medium while between 1.7% and 10% of ¹⁴C was detected in the cellular debris.

4. Discussion

Over the past 30 years, a considerable amount of information has accumulated in the literature regarding the bacterial degradation of PAHs. A number of reviews [2, 6, 7] have highlighted the catabolic diversity of bacterial PAH degradation, PAH degradative pathways, and application of

FIGURE 2: Pyrene mineralisation (a) by *Mycobacterium* sp. strains 1B (white square), BS5 (black diamond), KA5, (black square), KF4 (black triangle), and mercuric chloride killed controls (black circle). The distribution of ¹⁴C in the organic phase (undegraded pyrene: black square), aqueous phase (water soluble metabolites: Gray square), gaseous phase (¹⁴CO₂: white square), and cellular debris (dashed square) at the completion of mineralisation experiments is also shown (b).

these organisms to bioremediation strategies. It is apparent from these reviews and research in the scientific literature (e.g., [9, 18, 19]) that nocardioform bacteria, in particular *Mycobacteria*, play a crucial role in the biodegradation of PAHs. In this study, three pure cultures were recovered from three dissimilar inoculum sources (manufacturing gas plant soil, uncontaminated agricultural soil, and kangaroo faeces) following enrichment and isolation using PAH-containing media. All three organisms had the ability to utilise a variety of environmental contaminants as growth substrates

TABLE 4: Growth of *Mycobacterium* sp. isolates on various surfactants, phenols, hydrocarbons, and PAHs. Growth was scored visually on the basis of turbidity and ranked accordingly (see Table 3 for turbidity rankings).

Substrate	<i>Mycobacterium</i> sp. strain BS5	<i>Mycobacterium</i> sp. strain KA5	<i>Mycobacterium</i> sp. strain KF4
Surfactants			
Triton X-100	++	++	+
Tween 20	++	++	+
Tween 80	++	+	—
Tergitol NP-10	—	++	+++
Dowfax	—	—	—
Phenols			
3-chlorophenol, 4-chlorophenol, Cresol, Pentachlorophenol, 2,4,5-trichlorophenol	—	—	—
Hydrocarbons			
Benzene	++	++	++
Toluene	++	++	++
Xylene	+	++	+
Diesel, Heptane, Hexane, Hexadecane	+++	+++	+++
PAHs			
Acenaphthylene, Benzo[<i>a</i>]pyrene, Fluorene, Naphthalene	—	—	—
Phenanthrene	+++	+++	+++
Anthracene	++	—	+
Pyrene	+++	+++	+++
Fluoranthene	+	++	+
Benz[<i>a</i>]anthracene	++	++	++
Chrysene	—	++	+
Dibenz[<i>a,h</i>]anthracene	++	++	++

TABLE 5: PAH removal by *Mycobacterium* sp. isolates.

PAH ^a	Initial PAH concentration (mg L ⁻¹)	PAH Decrease (%) ^b					Incubation time (days)
		Strain 1B	Strain BS5	Strain KA5	Strain KF4	Abiotic control	
Fluorene	100	NS ^c	NS	NS	NS	52.9 ± 6.6	14
Phenanthrene	250	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	— ^d	14
Fluoranthene	50	100 ± 0.0	24 ± 3.8	59.6 ± 13.5	19.9 ± 3.9	—	20
Pyrene	250	100 ± 0.0	94.3 ± 6.2	100 ± 0.6	98.5 ± 2.6	—	20
Benzo[<i>a</i>]pyrene	50	NS	NS	NS	NS	—	50

^a PAHs were supplied in BSM containing 0.05 g L⁻¹ yeast extract.^b Percentage PAH decrease was calculated by comparing PAH concentrations in *Mycobacterium* sp. inoculated cultures to the corresponding abiotic control.^c PAH removal was less than 5% compared to the abiotic control and not significant, NS ($P > .05$). All other % removal values significantly greater than abiotic control ($P \leq .001$).^d Greater than 95% of added PAHs were recovered from abiotic controls at the end of the incubation period.

including surfactants, *n*-alkanes, mono-aromatic hydrocarbons, PAHs and complex hydrocarbon mixtures (diesel). By sequencing the 16S rRNA genes and utilising BLAST similarity search accessed through the NCBI database, the three isolates were identified as *Mycobacterium* species.

Phylogenetic analysis of the isolated *Mycobacterium* strains revealed that these isolates were related but not identical to other PAH-degrading *Mycobacterium* species.

Mycobacterium sp. strain BS5, isolated from a long-term PAH-contaminated soil (manufacturing gas plant

soil), aligned most closely to *Mycobacterium gilvum* and *Mycobacterium mucogenicum* isolates (98% similarity). The *Mycobacterium gilvum* isolates identified in the sequence database were obtained from environmental samples including polluted sediment (strain PYR GCK) [20], PAH-contaminated soil (strain BB1) [21] and uncontaminated forest soil (strain HE5) [22] following enrichment on PAHs or an aliphatic secondary amine (morpholine). Interestingly, *Mycobacterium* sp. strain BS5 was also closely aligned to *Mycobacterium mucogenicum* strain ATCC 49649, a rapidly growing clinically significant organism commonly recovered from tap water [23].

Unlike *Mycobacterium* sp. strain BS5, the other two isolates obtained in this study were recovered from samples that had no previous exposure to PAHs (agricultural soil and kangaroo faeces). *Mycobacterium* sp. strain KA5 was isolated from agricultural soil used for the cultivation of wheat and pea straw. This isolate was most closely aligned to *Mycobacterium monacense* B9-21-178 and environmental isolates *Mycobacterium* sp. strain KMS, and JLS (94% similarity). *Mycobacterium monacense* is a clinical isolate while *Mycobacterium* sp. strain KMS and strain JLS were isolated from soil collected from a former wood preserving facility (Champion International Superfund Site) in Libby, Montana, by their ability to mineralise pyrene [24].

Isolated from kangaroo faeces, *Mycobacterium* sp. strain KF4's 16S rRNA gene sequence was also most closely aligned (95% similarity) to *Mycobacterium* sp. strain KMS, strain JLS and *Mycobacterium monacense* strain B9-21-178, in addition, to *Mycobacterium* sp. strain MCS and *Mycobacterium vaccae* (VM0587 and VM0588). strain MCS was isolated from a former wood preserving facility [24] while it is unclear from where *Mycobacterium vaccae* strains were isolated.

Traditionally, the isolation of bacteria with specific catabolic activities has been confined to enumeration and isolation studies using soil containing the contaminant of interest [2]. Numerous PAH-degrading bacteria have been isolated from PAH-contaminated soil such as manufacturing gas plant soil, creosote-contaminated soil and alike [13, 19, 24–26]. The manufacturing gas plant soil used in this study contained high concentrations of PAHs even though operation of the plant ceased decades ago. Enrichment of *Mycobacterium* sp. strain BS5 from this soil on pyrene was not surprising given the long exposure time of the indigenous soil microorganisms to the PAHs and the relatively high proportion of pyrene in the soil (~10% of the total PAH concentration). It has been suggested that chronic exposure to petrogenic compounds may not increase the total number of heterotrophic microorganisms, however, it may selectively increase the hydrocarbon-degrading microbial population [25].

Two other *Mycobacterium* strains (KA5 and KF4) were isolated from environmental samples without previous exposure to PAHs. The propensity for strains KA5 and KF4 to degrade PAHs demonstrates that prior exposure to PAHs was not a prerequisite for PAH catabolic activity for these *Mycobacterium* isolates. Previous research has demonstrated that prior exposure to PAHs may be required for the induction of enzymes for PAH degradation [27–29]. It is

known that some enzymes involved in PAH degradation are inducible, being synthesised only when a particular metabolite or substrate is present [30]. However, Kim et al. [31] identified constitutive enzyme in *Mycobacterium vanbaalenii* PYR-1 responsible for PAH degradation. PAH quinone reductase and catechol-O-methyltransferase were demonstrated to be constitutive enzymes located in the soluble fraction of cell extracts.

All three *Mycobacterium* spp. isolated in this study possessed broad substrate specificities. *Mycobacterium* sp. strains BS5, KA5 and KF4 were able to utilise PAHs, alkanes, chlorinated phenols, monoaromatic compounds as well as possible PAH degradation products as sole carbon and energy sources. From a bioremediation viewpoint, this is a desirable feature since many PAH-contaminated sites contain a variety of organic pollutants. All three isolates were competent PAH degraders although subtle differences in their PAH-degrading profiles were observed. Whilst none of the isolates were capable of utilising the low-molecular-weight PAHs naphthalene, acenaphthylene, or fluorene as sole carbon and energy sources, strains BS5, KA5, and KF4 exhibited high-phenanthrene and pyrene-degrading efficacies.

¹⁴C-Pyrene experiments demonstrated the propensity for strains BS5, KA5, and KF4 to mineralise pyrene. When compared to a known pyrene degrader (*Mycobacterium* sp. strain 1B) [14], the extent of pyrene mineralisation was comparable for strains BS5, KA5, and KF4: following 10-day incubation between 70, and 79% of ¹⁴C-pyrene was converted to ¹⁴CO₂. Previous pyrene mineralisation studies in liquid medium inoculated with *Mycobacterium* sp. have reported 45–60% mineralisation of pyrene following 4 to 96 hours incubation [32–34]. Whilst the rate of pyrene mineralisation by *Mycobacterium* sp. isolated in this study was not as rapid as rates observed in the aforementioned studies, an enhanced extent of pyrene mineralisation was observed for *Mycobacterium* sp. strains BS5, KA5, and KF4. Presumably, the lag period preceding pyrene mineralisation and the resulting decreased rate of pyrene mineralisation was attributable to the smaller inoculum size used in this study.

At the completion of mineralisation experiments, less than 3% of ¹⁴C was detected in the culture medium, indicative of the low amount of polar metabolites produced by *Mycobacterium* sp. strains BS5, KA5, and KF4. In contrast, following pyrene mineralisation experiments conducted with *Mycobacterium* sp. strain RJGII-135 [34], 79% of the total organic extractable residue comprised four pyrene metabolites (4-phenanthrene carboxylic acid, 4,5-phenanthrene dicarboxylic acid, 4,5-pyrene dihydrodiol and unidentified PYR-IV). Microorganisms with the ability to mineralise PAHs are advantageous for bioremediation applications as they minimise the accumulation of potentially problematic transformation products. Some PAH metabolites are genotoxic and have been shown to produce DNA strand scission, DNA adduct formation, and malondialdehyde generation [35]. In addition, the increased polarity of PAH transformation products, compared to the parent compound, results in their enhanced mobility in the environment which may culminate in potential impacts on ecological and groundwater receptors.

Previous reports have demonstrated *Mycobacterium* isolates capable of degrading pyrene and other 4 ringed PAH. Many of these reports have demonstrated isolation and enrichment from previously contaminated sites, some as a direct substrate and other cometabolically [14, 18, 20, 21]. The literature indicates that environmental *Mycobacteria* spp. are well adapted to degradation of relatively hydrophobic molecules, most probably this characteristic is associated with the hydrophobic nature of their cell wall structure [7].

This paper demonstrated the ubiquity of *Mycobacteria* spp. in soil environments with the ability to degrade PAH. That two of the isolates were derived from uncontaminated material is of interest, since the majority of published PAH-degrading isolates have come from contaminated sites [2, 6, 8]. This observation points to the broad exposure of organisms to aromatic compounds such as lignin and tannins as a plentiful carbon source in a diverse range of soil systems [8, 10]. In contrast to the report of Cheung and Kinkle [18] pristine soils with naturally elevated aromatic content may therefore be an alternative source of PAH-degrading organisms that might assist in bioremediation projects.

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