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Assessing the degradation efficacy of native PAH-degrading bacteria from aged, weathered soils in an Australian former gasworks site

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ABSTRACT

The large-scale degradation of polycyclic aromatic hydrocarbons (PAHs) by bacteria represents a promising soil remediation strategy. The aim of this study was to identify bacterial isolates for potential use in the remediation of PAH-contaminated sites using selective isolation plating, Biolog™ MT2 evaluation and metabolic profiling on the Biolog™ system. Thirty-five bacterial isolates were isolated from aged, weathered PAH-contaminated soil using Bushnell Hass medium amended with phenanthrene as the sole carbon source. Selected isolates that were then able to grow on at least one of three model PAHs (naphthalene, phenanthrene and pyrene) were identified and then screened for their potential application in the bioremediation of aged, weathered soils from a former gasworks site based on Biolog™ MT2 data. The 16S rRNA gene sequencing showed that hydrocarbon degrading isolates were affiliated to *Rhodococcus* sp., *Achromobacter* sp., *Oerskovia paurometabola*, *Pantoea* sp., *Sejongia* sp., *Microbacterium maritypicum* and *Arthrobacter equi*. Enzyme studies confirmed catechol 1,2-dioxygenase activity in all of the isolates. Biolog™ Eco plates, applied in this study to evaluate the metabolic properties of seven isolates showed that all of these isolates could use a wide range of organic substrates, *Sejongia* sp. being the highest (28 of 31 substrates). Furthermore, the metabolic patterns of seven isolates on different substrates were summarized according to the biochemical categories of the substrates present on Biolog™ Eco plates. The isolates showed diverse performance on different biochemical categories (amino acids, phenolic compounds, amines, carbohydrates, carboxylic acids and polymers). An insight into the mechanisms by which selected bacteria degrade model PAHs and survive in nitrogen deficient soils was obtained. This study demonstrates the functional potential of indigenous bacteria for model PAHs-degradation and bioremediation of aged, weathered PAH contaminated sites of Australia.

1. Introduction

Sites surrounding gas manufacturing plants have frequently been contaminated with different fractions of polycyclic aromatic hydrocarbons (PAHs) (Thomas and Lester, 1993). PAHs represent a group of organic compounds containing two or more fused aromatic rings with only carbon and hydrogen atoms in cluster, angular or linear arrangements. They occur naturally in gasoline, coal and crude oil. PAHs emitted to the atmosphere from various industries accumulate in soils via dry and wet deposition (Lee and Lee, 2004). PAHs are of great concern because of their abundance, persistence, toxic effects and their lipophilic, mutagenic and carcinogenic properties (Abdel-Shafy and Mansour, 2016). PAHs represent the first recognized environmental carcinogens (Balachandran et al., 2012). Various environmental protection agencies have listed sixteen PAHs as priority pollutants in both aquatic and terrestrial ecosystems. Globally, 0.5 million tonnes (16 priority PAHs) were reported to be emitted into the atmosphere in 2007 by various sources (de Boer and Wagelmans, 2016; Shen et al., 2013). In Australia, around 30 to 40% of contaminated sites (up to 80,000) are mainly polluted by hydrocarbon contaminants, many containing PAHs (Asquith et al., 2012). PAHs pose a serious threat by exhibiting ecotoxicological activity as they are retained in the system for a long time and have the capability to impair ecosystem function which may take years to recover (Maliszewska-Kordybach et al., 2007).

Remediation of industrial sites can be expensive and challenging for many environmental and technical reasons including weather conditions, site location and the amounts and complexity of waste residues. However, a number of remediation technologies including biological,

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physico-chemical and thermal processes are available to reduce the PAH-concentrations in soil and also to reduce the risk associated with the exposure to these chemical compounds in various ecosystems (Sannino and Piccolo, 2013).

Bioremediation offers a number of advantages over conventional technologies. The ability to degrade PAHs is widespread among microorganisms and bioremediation has proven to be one among the most effective, environmental friendly, reliable and economical ways to remove these PAHs from field-contaminated soil (Xu et al., 2015). However, degradation of high molecular weight PAHs is very extensive than removal of low molecular weight PAHs (Chen and Aitken, 1999). Indeed a large number of novel microorganisms including bacteria and fungi have been screened, isolated and documented from PAH-contaminated sites with the ability to degrade PAHs efficiently. Bacteria of various genera including *Mycobacterium*, (Boldrin et al., 1993; Gong et al., 2015; Rehmann et al., 1998) *Rhodococcus*, (Dean-Ross et al., 2001; Song et al., 2011) *Sphingomonas*, (Guo et al., 2010) *Pseudomonas*, (Isaac et al., 2013; Safahieh et al., 2014; Sopeña et al., 2014), *Micrococcus*, (Ifeanyi and Ihenatuoha, 2014; Kumar et al., 2011) *Corynebacteria*, (Kafilzadeh and Pour, 2012; Kamil et al., 2013) *Nocardia*, (Zeinali et al., 2008) *Acinetobacter*, (Shao et al., 2015; Yuan et al., 2014) and *Burkholderia* (Andreolli et al., 2011; Revathy et al., 2015) are identified as PAH-degraders (Mahjoubi et al., 2013). However, contaminant aging can decrease the fraction of organic contaminants available for biodegradation based on their molecular weight and therefore reduce the efficiency of biodegradation (Chen and Aitken, 1999; Ling et al., 2010). The uniqueness of Australian soils and environmental conditions complicate the use of exogenous bacterial isolates that have been previously shown to degrade PAHs in other polluted sites. For instance, contaminated soils in parched locales of Australia generally lack adequate soil nutrients that are required for the degradation of the hydrocarbons by microorganisms. Additionally, the soil moisture content may not be adequate to maintain the degradation of the hydrocarbon contaminants throughout the year as a result of extreme weather conditions (Smith et al., 2015). Furthermore, contaminant aging can decrease the fractions of organic contaminants available for biodegradation and reduce the efficiency of biodegradation (Ling et al., 2010). Therefore, the development of a successful bioremediation strategy is highly dependent upon the screening and identification of indigenous microorganisms that efficiently degrade PAHs. These organisms may have additional factors (e.g. biosurfactant production; heavy metal tolerance, efficient nitrogen utilization) that make them more efficient degraders in any given environment. The nitrogen content of PAH-contaminated soils is generally low and nitrogen has been shown to be a major limiting factor in bioremediation (Rosenberg et al., 1992). Studies to specifically examine the organisms that are active in incorporating nitrogen sources and their potential hydrocarbon degradation are limited. The aim of the present study was to isolate PAH-degrading bacteria for potential use in the bioremediation of weathered PAH-contaminated soil via selective isolation plating on media containing model PAHs as sole carbon and energy source. Subsequently, these strains were identified and their PAH-degrading capabilities were investigated using Biolog™ assays.

2. Materials and methods

2.1. Soil collection and characterization

Soil samples were collected from an aged stockpile of soil with a history of long term contamination of PAHs from a former gas work contaminated site located in Cootamundra, New South Wales, Australia (34°31′26.6″S 147°35′56.6″E). Contaminated soil (25 kg) was collected, labelled and transported to the research facility and stored at 4 °C prior to further analysis. Soil samples were air dried at room temperature for one day before use. Soils were sieved initially through a 2 mm sieve to remove larger particles including gravels and debris and then ground using a pestle and mortar. Soil:water suspension (1,2 *w/v*) was used to measure the soil pH. All samples were analysed for various physico-chemical parameters (Table 1) using the standard methods described by Boll et al. (2015) and Rayment and Lyons (2011).

Table 1
Physico-chemical properties of the contaminated soils from the former Cootamundra gas works.

Properties	Values
Sand (%)	62
Loam (%)	20
Clay (%)	18
pH	7.8 ± 0.2
Soil moisture content (%)	10.4
Maximum water holding capacity (%)	45.8
Total carbon (%)	6
Total nitrogen (%)	0.28
Total phosphorous (%)	0.12
Soil Porosity (%)	36

2.2. PAH extraction from contaminated soil and analysis

PAHs were extracted by rapid solvent extraction method (n = 3) and dried as described previously using acetone:hexane by the authors (Haleyur et al., 2016). The total and individual PAH-composition and concentrations in the soil were determined by GC–MS/MS as described previously (Haleyur et al., 2016). Hexane was used as the injection solvent to improve the separation of fractions as it is a nonpolar solvent, as are PAHs.

2.3. Isolation of model PAH-degrading bacteria

Bushnell Hass (BH) mineral salts medium (Bushnell and Haas, 1941) enhanced with phenanthrene as the sole carbon source (Sigma Aldrich, NSW, Australia) was used to isolate bacteria from the contaminated soils. Bushnell Hass agar plates, supplemented with phenanthrene (1% (*w/v*)) as substrate, were inoculated with diluents of soil stored at 4 °C using the spread plate method (Bushnell and Haas, 1941). Plates were incubated at 28 °C for 6 days. Colonies were differentiated initially on the basis on their colony morphologies (shape, size, margin, elevation, texture and pigmentation). Further transfers of the observed colonies were carried out by sub-culture onto BH agar plates sprayed with three model PAHs: naphthalene, phenanthrene and pyrene (1% (*v/v*)). The plates were incubated at 28 °C to confirm growth on the substrate as sole carbon and energy source. Bacterial strains exhibiting a clear zone upon utilization of model PAHs were subcultured onto nutrient agar plates.

2.4. Carbon source utilization testing using Biolog™ MT2 plates

A loopful of re-streaked purified bacterial cultures from nutrient agar plates were inoculated into 20 mL of nutrient broth and incubated at 28 °C by shaking at 120 rpm for 24 h. Cultures with optical density (OD) values of 0.77–1.60 at 600 nm were harvested by centrifugation at 16,000 × *g* for 2 min. The pellets were washed twice with sterile distilled water and the concentrations were normalised (OD - 0.3) according to Kadali et al. (2012). Cell pellets were then resuspended in 2 mL of sterile distilled water prior to use in Biolog™ MT2 plates.

Three different PAH-compounds; naphthalene (NAP), phenanthrene (PHE) and pyrene (PYR) (Sigma-Aldrich) were selected for use in Biolog™ MT2 plates. The substrates were representative of different aromatic fractions containing increasing numbers of aromatic rings. The selected substrates were dissolved in acetone (final concentration of 2% (*v/w*)) and comparison between the isolates able to degrade the different PAHs was carried out using 96 well Biolog™ MT2 plates (Kadali et al., 2012). An aliquot (15 µL) of 2% (0.3 mg of each PAH in

15 μL) stock solution of individual PAHs were pipetted into cold Biolog™ MT2 plates (maintained on ice) in a fume hood. Plates were left on ice after loading the substrates for 30 min to enable the acetone to evaporate. Resuspended bacterial pellets (150 μL) with an OD of 0.3 were inoculated into individual wells on the Biolog™ MT2 plate containing each substrate. Aliquots (150 μL) of resuspended bacterial pellets were also inoculated into control wells without any PAH-substrate and containing only acetone. Plates were placed in a tray containing wet paper towels to avoid desiccation of wells and incubated at 28 °C in the dark with the lids closed. Each individual bacterial isolate was tested in triplicate on the three different hydrocarbon compounds. Readings were taken every 2 h for the first 48 h, then every 12 h thereafter up to day 5. Colour formation in Biolog™ MT2 plate wells were analysed using a multiscan microplate reader (Labsystems, Finland, Multiskan EX version 1.0).

The absorbance readings of control and individual wells were subtracted from the corresponding 0 h readings. The absorbance for substrates only at each time point was calculated by subtracting the control wells' OD values from the substrate wells' OD values. The average OD values for all the bacterial test isolates were calculated at each time point up to day 5. Cluster analysis was carried out on data obtained from Biolog™ MT2 to classify bacterial isolates at substrate-specific time intervals (NAP - 3 h, PHE - 1.5 h, PYR - 24 h) (Kadali et al., 2012).

2.5. DNA extraction, PCR amplification and identification

DNA was extracted from the bacterial cells grown in 10 mL nutrient broth for 24 h. Cells were obtained by centrifugation at 4 °C at 16,000 × *g* for 2 min. The pellets were washed with sterile distilled water, and DNA was extracted using a MoBio Power Soil DNA isolation kits (Mo Bio Laboratories, Inc., USA) in accordance with the manufacturer's instructions.

The 16S rRNA gene sequences were amplified from DNA of individual isolates using primers 63F (5′CAGGCCTAACACATGCAA GTC-3′) and 1389R (5′ ACGGGCGGTGTGTACAAG-3′) (Osborn et al., 2000). The amplified DNA products were purified using a PCR clean up kit (Wizard SV PCR clean-up system, Promega, USA) and quantified with Nanodrop (Thermoscientific, USA). PCR products were sequenced at the Australian Genome Research Facility (AGRF, Melbourne, Australia). Sequences were assembled and edited using Sequencher (version 5) and compared to sequences present in the GenBank using the BLASTn site at NCBI server (Altschul et al., 1990). Alignment of DNA sequences was done using the CLUSTALW program using MEGA 7 (Kumar et al., 2016). The partial 16S rRNA gene sequences of all the isolates are deposited in the GenBank database under Accession numbers: MF440325 - MF440331.

2.6. Enzyme assays

To prepare crude cell free extracts, bacterial isolates were grown in BH broth (100 mL) supplemented with phenanthrene for two days and later harvested at the mid exponential phase of growth by centrifugation at 4500 × *g* for 15 min at 4 °C. Cell pellets were washed twice with phosphate buffer saline (PBS) to remove any nutrients/salts present and centrifuged after each washing with similar conditions. Cells were resuspended using 1 mL of lysis buffer that contained 5 mM ammonium sulphate, 50 mM Tris-HCl [pH 7.5], 1 M glycerol, 1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT and sonicated 10 times on ice for 10 s (Erdoğmuş et al., 2013). The suspensions were centrifuged at 10,000 × *g* for 15 min at 4 °C and the supernatants were used as crude cell free extract for protein estimation and enzyme assays. Protein concentration was determined following the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976). Ring fission enzymes were detected as described previously (Obayori et al., 2008).

2.6.1. Catechol 1,2 dioxygenase and catechol 2,3 dioxygenase assays

Catechol 1,2 dioxygenase activity was measured spectrophotometrically at UV - 260 nm using a 96 well UV plate to detect the product cis, cis muconate (pH = 7.0) (Hegeman, 1966). Catechol 2,3 dioxygenase activity was detected by measuring the rate of increase in absorbance at 375 nm due to the formation of 2-hydroxyl muconic semi aldehyde (Klecka and Gibson, 1981). Specific enzyme activity was reported as 1 μmol min^{-1} mg^{-1} of protein.

2.7. Metabolic profiling using Biolog™ Ecoplates

The capability of bacterial isolates to utilize thirty one different carbon sources was tested in triplicate on Biolog™ Eco plates (Thomas et al., 2016). Plates were placed in a tray containing wet paper towels to avoid desiccation of wells and incubated at 28 °C in the dark with the lids closed. The intensity of colour change of the tetrazolium dye was measured with an optical density multiscan microplate reader (Labsystems, Finland, Multiskan EX version 1.0) at 595 nm over 4 days at t = 0, 24, 48, 72, 96 h (Merkl and Schultze-Kraft, 2006). OD values obtained for each substrate well were corrected by subtracting the blank well values. OD values higher than 0.2 after 96 h of incubation were defined as positive utilization of a carbon source. The average absorbance values for all of the bacterial isolates were calculated at each time point. The average well colour development (AWCD) over time was calculated for each isolate using the formula $AWCD = \Sigma (A_{substrate} - A_{blank}) / 31$, where $A_{substrate}$ is the absorbance of each carbon source-containing well and A_{blank} is the absorbance of the negative control well. The average absorbance after 96 h in each biochemical category (amine, amino acids, carbohydrate, carboxylic acids, phenolic compounds and polymers) was calculated and compared between the categories to determine which type of organic substrates are more likely to be utilized by bacterial isolates.

2.8. Statistical analysis

Biolog™ experimental data are presented as means of three independent measurements (three replicates for each bacterial isolate). Analysis of variance (one way ANOVA) was performed on data using the Minitab statistical analysis program. Independent samples t-tests were used to compare any significant differences between the different isolates. Mean values were compared using the Least Significant Difference (LSD) test ($P \leq 0.05$), where the F-value was significant. The standard errors (SE) are reported, where required.

3. Results and discussions

3.1. Physico-chemical characteristics of the soil

The Cootamundra industrial plant operated for a long period of time and heavy industrial use of this area has resulted in high PAHs contamination. Various physico-chemical characteristics of the soil sampled from the former gaswork site are presented in Table 1. The C:N:P ratio in the soil was found to be 50:2:1 indicating that the soil was nitrogen deficient and phosphorus rich when compared with the standard “Redfield ratio” in soil (60:7:1) (Cleveland and Liptzin, 2007). In PAH-contaminated soil systems, nitrogen is mostly deficient compared to carbon as carbon is a major constituent of hydrocarbons that includes PAHs.

The total concentration of the 16 priority pollutants (ΣPAHs) listed by the United States Environmental Protection Agency (USEPA) was 716 ± 46.37 mg kg^{-1} (Table 2), and individual PAHs had concentrations ranging from 5 ± 0.24 mg kg^{-1} to 100 ± 9.61 mg kg^{-1} soil (dry weight) (Table 2). The concentration of fluoranthene was highest (100 ± 9.61 mg kg^{-1}) (Table 2). The concentration of benzo(*a*)pyrene (BaP) was found to be 80 ± 3.51 kg^{-1} (Table 2) which was 16 fold higher than the acceptable guidelines of National Environmental

Table 2
Concentration of 16 USEPA individual and ΣPAHs in the contaminated soils determined by GC–MS/MS. ± indicate the standard deviation. The values are compared with another similar site study at France (Potin et al., 2004).

PAHs	No. of rings	PAH concentration mg kg^{-1} (dry weight)	Percentage of total PAH	Percentage of total PAH of a gas manufacturing site at France (Potin et al., 2004)
Naphthalene	2	5 ± 0.24	0.7	0.2
Acenaphtylene	3	15 ± 3.95	2.09	0.14
Acenaphtene	3	9 ± 0.71	1.26	0.1
Fluorene	3	7 ± 1.01	0.98	0.37
Phenanthrene	3	46 ± 6.03	6.42	2.82
Anthracene	3	18 ± 2.26	2.51	2.77
Fluoranthene	4	100 ± 9.61	13.97	10.94
Pyrene	4	91 ± 7.64	12.71	8.56
Benzo(*a*)anthracene	4	55 ± 3.45	7.68	9.32
Chrysene	4	51 ± 3.31	7.12	9.54
Benzo(*b*)fluroanthene	5	69 ± 3.60	9.64	ND
Benzo(*k*)fluoranthene	5	56 ± 2.70	7.82	12.76
Benzo(*a*)pyrene	5	80 ± 3.51	11.17	16.8
Indeno(1,2,3-*cd*)pyrene	5	41 ± 1.87	5.73	4.77
Dibenzo(*a*,*h*)anthracene	6	19 ± 1.23	2.65	11.3
Benzo(*g*,*h*,*i*)perylene	6	54 ± 2.40	7.54	9.53
Total 3-ring PAHs		95	13.27	6.22
Total 4-ring PAHs		297	41.48	38.38
Total 5,6–ring PAHs		319	44.55	55.18
ΣPAHs		716 ± 46.37		793.92 mg kg^{-1}

Protection Council with threshold limits of BAP set at 5 mg kg^{-1} of soil (NEPC, 1999). Total PAH-concentrations were found to be similar (793.92 mg kg^{-1}) to those at an aged contaminated site in France (Table 2) (Potin et al., 2004). The percentage of 2-ring compounds was found to be low (< 1%), indicating that readily degradable compounds had already been removed from the soil (Table 2). However, 3 ring (13.2%) and 4 ring (41.4%) compounds were found in higher concentrations and 5, 6 ring compounds (44.5%) were found in lower concentrations at the Cootamundra site compared to the other study site in France (Table 2) (Potin et al., 2004). In another study conducted by Thavamani et al. (2012) at a former manufactured gas plant site in New South Wales, Australia, total PAHs ranged from 335 to 8645 mg kg^{-1} confirming similar levels of contamination in most samples compared to our study. The levels of PAHs represent a good source of carbon and energy to the hydrocarbonoclastic bacterial community. This site in Australia is characterised by a lowest temperature of −7.6 °C during winters and highest temperatures reaching 45 °C, with mean annual rainfall of 598.1 mm. These conditions may lie beyond those in which mesophilic strains can adapt. Hence, members of the indigenous microbial community capable of degrading PAHs that already exist in contaminated soils may prove to be effective compared to commercially available strains.

3.2. Isolation, screening and identification of PAH-degraders

Thirty-five isolates grew at 28 °C on BH selective plates supplemented with phenanthrene (1% *v/v*) as sole carbon and energy sources. Previous studies have shown that the presence of clear zones around the bacterial isolates growing on PAH-substrates are indicative of PAH-degradation (Ahmed and Ahmed, 2016). The clear-zone diameter method was applied to all of the 35 isolates that were able to grow on BH plates using naphthalene, phenanthrene and pyrene as sole carbon source. Naphthalene has the simplest structure and highest solubility of the three PAHs and is most soluble in nature and as a consequence has long been used as a model compound in PAH-bioremediation studies (Goyal and Zylstra, 1997). In addition, phenanthrene is often used as a model substrate due to the presence of bay-and K-regions similar to more toxic benz[*a*]anthracene, benzo[*a*]pyrene and chrysene (Samanta et al., 1999). Pyrene is used as the model substrate to study the metabolism of high molecular weight PAHs and is found in relatively high concentrations in coal tar-contaminated sites and was present at high concentrations in this study (Table 2) (Seo et al., 2009). Based on the results obtained from the use of the clear-zone formation method on BH plates (supplementary Table 2), only 22 bacterial isolates (62%) were selected according to their capability to utilize naphthalene, phenanthrene and pyrene as sole carbon and energy sources (with zones of clearance between 0.8 and 1.2 cm in diameter).

Partial bacterial 16S rRNA gene sequences of all 22 bacteria showed a match of 98–100% to those from bacteria in the GenBank database including members of the phyla:Actinobacteria (13 isolates), Proteobacteria (7 isolates), Bacteroidetes (1 isolate) and Firmicutes (1 isolate). The predominance of Actinobacteria and Proteobacteria in the soils studies was expected, because those groups have been described as common inhabitants of PAH-contaminated soils (Zhang et al., 2011). Our results are consistent with the study conducted by Niepceron et al. (2013) which showed that higher numbers of proteobacteria are present in PAH-contaminated soils; in fact these authors suggested that proteobacteria can be used as potential bioindicators of biodegradation (Niepceron et al., 2013). The other reason for the increased number of proteobacteria could be the presence of clay particles (18%) in this soil (Table 1); the presence of clays are often linked with the presence of a large proteobacterial community (Ding et al., 2013).

Quantitative degradation of model PAHs by individual isolates could not be assessed using growth on agar plates. Biolog™ MT2 plates designed on tetrazolium chemistry have been successfully used for screening and evaluation of bacterial strains growing on hydrocarbons (Kadali et al., 2012). Hence the ability of each of these isolates to utilize three different PAH-fractions (naphthalene, phenanthrene and pyrene) were profiled using Biolog™ MT2 plates (Kadali et al., 2012; Mansur et al., 2014). All 22 bacteria showed higher capabilities of utilizing naphthalene, with 16 bacteria also showing elevated capacity to utilize phenanthrene and 15 bacteria showing greater capacity to utilize pyrene (Fig. 1). The substrate on which growth was highest (greatest degradation) as measured by maximum average absorption (595 nm) was naphthalene confirming lower molecular weight PAHs tend to be oxidized more rapidly compared to higher molecular weight PAHs. Previous studies showed that 3 ring PAHs such as phenanthrene had the higher bioavailability and are less hydrophobic than the high-molecular-weight compounds (e.g. - pyrene) (Heitkamp and Cerniglia, 1987). The main reason could be bioavailability as not all PAHs are bioavailable or may be limited (Reid et al., 2000). All model PAHs were utilized over the first two days suggesting that the degradation was complete (Fig. 1). All isolates showed higher rates of degradation and fast utilization of model PAHs when compared with the results reported

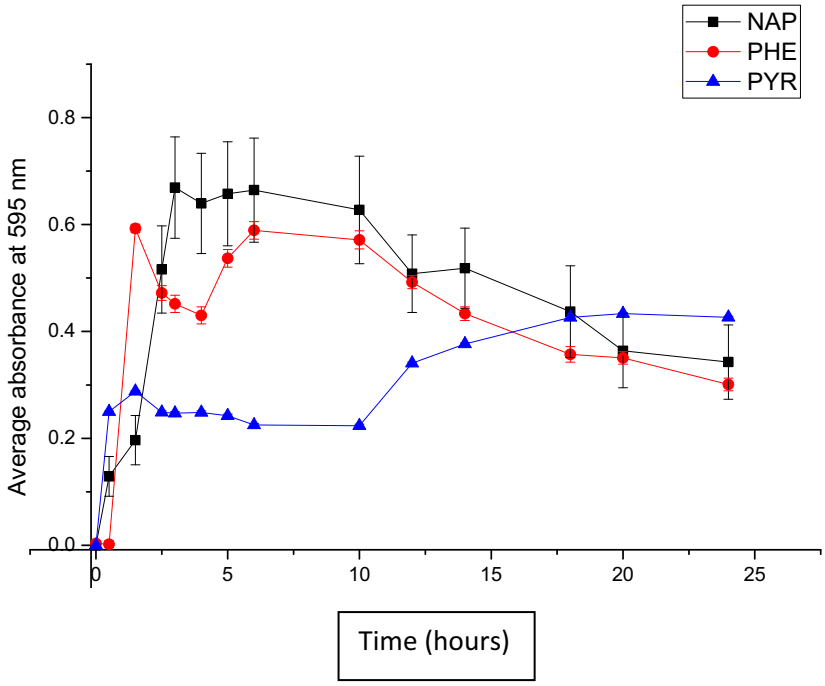

Fig. 1. Growth (average colour development on Biolog™ MT2 plates) by 22 bacterial on three different polyaromatic hydrocarbons over 24 h. Error bars show ± standard error (n = 22) at each time point.

by Mansur et al. (2014). The ability of the isolates to grow at higher concentrations of model PAHs, up to 2% is noteworthy.

The isolates with the greatest model PAH-degradation activity based on cluster analysis (data not shown) included: five isolates of *Rhodococcus* sp., four isolates of *Achromobacter* sp., and one isolate of each of *Oerskovia paurometabola* (NH11), *Pantoea* sp. (NH15), *Sejongia* sp. (NH20) *Microbacterium* sp. (NH30) and *Arthrobacter equi* (NH21). *t*-Test and ANOVA confirmed that there were no significant differences in substrate utilization abilities between the five isolates of *Rhodococcus* sp. and similarly between the four isolates of *Achromobacter* sp. (data not shown). One representative of each of the isolates, NH2 and NH13 for *Rhodococcus* sp. and *Achromobacter* sp. respectively were selected for further studies. A total of seven organisms were selected for further experiments and the details are outlined in Table 3. The abundance of *Achromobacter* sp., (Dave et al., 2014; Ma et al., 2015) and *Rhodococcus* sp. (Dean-Ross et al., 2001; Song et al., 2011) in the studied soil denote the ability of members of this genus to grow in soils contaminated by PAHs. Previous research suggests that *Pantoea* sp., *Microbacterium* sp. (Fernet et al., 2016) and *Arthrobacter* sp. (Thion et al., 2013) are excellent PAH-degraders. However, *Arthrobacter equi* has not been previously reported as PAH degrader, although this capability is common in the phylogenetically closely related *Arthrobacter oxydans* (Thion et al., 2013). The selected isolates showed high levels of PAH-degradation capability on Biolog™ MT2 plates. To our knowledge this is the first study reporting *Sejongia* sp., *Arthrobacter equi* and *Oerskovia paurometabola* as model PAH-degraders. However, their capability to degrade other PAHs present in contaminated soils requires further study.

3.3. Detection of PAH-degrading enzymes

The degradation pathway of PAHs mostly entails the addition of two atoms of oxygen onto the aromatic ring and subsequent cleavage of the dihydroxylated compound either by *ortho* or *meta* cleavage (Cerniglia, 1993). The cell suspensions of all of the isolates tested for ring fission enzymes failed as yellow colouration was not observed (grown on both phenanthrene and catechol) in the presence of catechol, indicating the absence of meta-cleavage pathways of catechol. The appearance of purple colour upon testing for β-keto-adipic acid showed ortho-cleavage pathway in all the isolates (Table 3).

The specific activities of catechol 1,2-dioxygenase and catechol 2, 3 dioxygenase obtained for all of the isolates are presented in Table 3. *Achromobacter* sp., had the highest catechol 1,2-dioxygenase activity and *Oerskovia paurometabola* and *Achromobacter* sp. had the lowest catechol 1,2-dioxygenase activity (Table 3). The presence of catechol 1,2-dioxygenase involved in *ortho* cleavage pathway among the degradative

Table 3
Taxonomic identities, specific enzyme activity (means ± SE; n = 3) and degradation pathway of selected bacterial species isolated from aged, PAH contaminated soil. * Catechol 2,3 dioxygenase was not detected.

Isolate	Phylum/class	Identification	% identity	Accession number of closest related isolate	Specific enzyme activity (1 μmol min^{-1} mg^{-1})	Cleavage pathway
					Catechol 1,2 dioxygenase	
NH2	Actinobacteria	*Rhodococcus* sp.	100	MF440325	1.65 ± 0.01	Ortho
NH11	Actinobacteria	*Oerskovia paurometabola*	100	MF440326	1.34 ± 0.04	Ortho
NH13	Proteobacteria	*Achromobacter* sp.	99	MF440327	9.74 ± 1.2	Ortho
NH15	Proteobacteria	*Pantoea* sp.	100	MF440328	7.76 ± 0.03	Ortho
NH20	Bacteroidetes	*Sejongia* sp.	99	MF440329	7.41 ± 0.03	Ortho
NH21	Actinobacteria	*Arthrobacter equi*	100	MF440330	1.34 ± 0.01	Ortho
NH30	Actinobacteria	*Microbacterium* sp.	100	MF440331	4.39 ± 0.1	Ortho

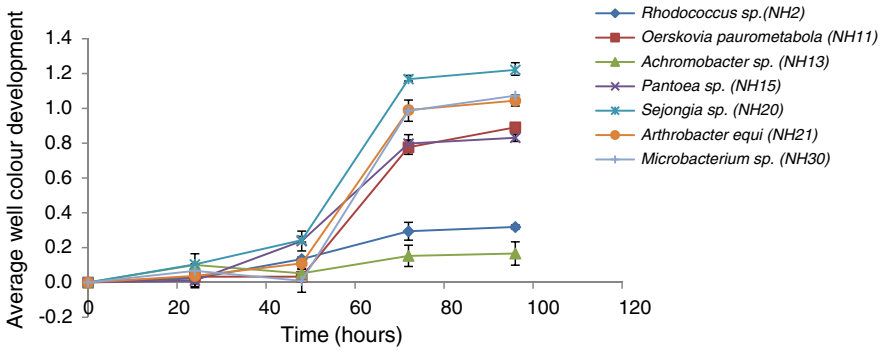

Fig. 2. Average well colour development over time for seven bacterial isolates on Biolog™ ECO plate at 28 °C monitored for 96 h. Average well colour development is derived from the mean difference among absorbency values of all the sole carbon substrates and the water blank. Error bars show ± standard error (n = 3) at each time point.

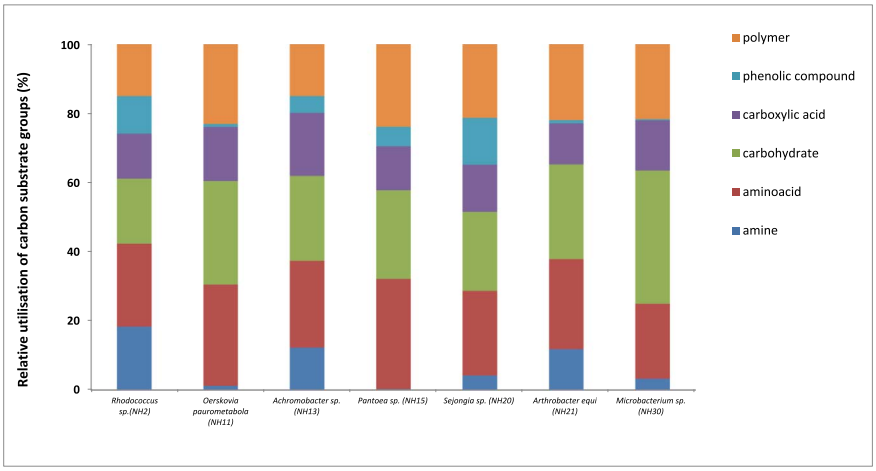

Fig. 3. Relative carbon source utilization by seven bacterial isolates on six substrate groups (amine, amino acids, carbohydrate, carboxylic acids, phenolic compounds and polymers) on Biolog™ EcoPlates.

bacteria is widespread (Harwood and Parales, 1996) and the use of one cleavage pathway or the other is dependent upon the bacterial species and type of growth substrate. Consistent with our findings, Song (2009) reported that all isolates used in their study that were obtained from pine litter were able to grow in the presence of PAHs and degraded catechol using the *ortho* cleavage of the β ketoadipate pathway (Song, 2009). Obayori et al. (2008) also showed that only catechol 1,2-dioxygenase activity was detected in isolates obtained from hydrocarbon contaminated sites in Nigeria.

3.4. Metabolic profiling

Biolog™ EcoPlates have been used to evaluate the functional diversity of whole communities in various environments including soil, water, activated sludge, compost, wastewater, and industrial wastes. This approach, often called community-level physiological profile (CLPP) has been demonstrated to be effective in evaluation of changes in microbial communities structure and function. Additionally, Chojniak et al. (2015) demonstrated the use of Biolog™ EcoPlates for evaluation of physiological profiling for a single isolate of *Serratia marcescens ss marcescens* isolated from onsite wastewater technology. In the current study, the seven selected bacterial isolates were subjected to individual physiological profiling using Biolog™ EcoPlates that contained 31 different carbon sources. The numbers of utilizable substrates in the Biolog™ EcoPlates plate for each of the isolate tested are shown in Supplementary Table 1. *Sejonjia* sp. (NH20) was capable of utilizing 28 (90.3%) of the organic substrates in the Biolog™ EcoPlates. *Pantoea* sp. (NH15) and *Arthrobacter equi* (NH21) could metabolize 26 (83.9%) substrates; *Rhodococuus* sp., (NH2) *Oerskovia paurometabola* (NH11) and *Achromobacter* sp. (NH13) were able to metabolize 23 (74.2%) substrates whilst *Microbacterium* sp. could utilize 22 (71%). The results revealed that all isolates could oxidize a wide-range of organic substrates. However, none of the isolates tested were able to utilize Itaconic acid as the sole carbon source.

Average well colour development (across all substrates) for all the isolates on Biolog™ EcoPlates significantly increased, demonstrating the capacity to metabolize the organic substrates in Biolog™ EcoPlates. Over the 100 h incubation period for all of the isolates tested, *Sejongia* sp. showed the most intense metabolic response (Fig. 2), with maximum colour development (A = 1.22 ± 0.041). *Achromobacter* sp. showed the lowest average metabolic rate for the substrates in the Biolog™ EcoPlates with A = 0.17 ± 0.06 (Fig. 2). Catabolic diversity among different organisms was compared using Pielou's evenness (J) and Shannon diversity index at 96 h (Supplementary Table 1). *Sejongia* sp. showed highest Shannon diversity index and Pielou evenness (Supplementary Table 1).

Fig. 3 shows the percentage utilization of the six substrate categories by the seven organisms on Biolog™ EcoPlates. The isolates showed diverse utilization on different categories of substrates. *Sejongia* sp. (NH20) and *Rhodococcus* sp. (NH2) utilized substrates from all of the six categories (Fig. 3). Two isolates, *Oerskovia paurometabola* (NH11) and *Pantoea* sp. (NH15) could not oxidize amines on Biolog™ EcoPlates (Fig. 3). Decreases in the affinity for amines and increases in the consumption of polymer and amino acids can indicate that the isolates are

involved in the metabolism of recalcitrant complex molecules (Ros et al., 2014). Three isolates, *Oerskovia paurometabola* (NH11), *Arthrobacter equi* (NH21), *Achromobacter* sp. (NH13) and *Microbacterium* sp. (NH 30) could not utilize phenolic compounds. Amino acids, carbohydrates, carboxylic acids and polymers were utilized by all of the isolates. The higher utilization of recalcitrant polymers in Biolog Eco™ plates confirms the metabolism of recalcitrant complex molecules by the isolates. Likewise, the high consumption of carboxylic acids might indicate that isolates metabolize mainly organic substances such as hydrocarbons (Kreitz and Anderson, 1997). As previously described, nitrogen deficiency compared to carbon in PAH-contaminated soils may lead to higher utilization of organic nitrogen (amine/amides and amino acids) on Biolog Eco™ plates. This technique of physiological profiling of individual isolates might also find application in searching for similarities and differences in the functional diversity of microbial isolates in different aged, contaminated PAH-soils. This study shows the potential for the application of seven isolates in bioaugmentation of aged, weathered nutrient (nitrogen) deficient soils.

4. Conclusions

This study investigated the availability of hydrocarbonoclastic bacteria in aged Cootamundra soils and the ability of selected isolates to utilize model PAH-substrates. Multiple genera were identified that could conduct model PAH-degradation. This is the first report identifying *Sejongia* sp., *Arthrobacter equi* and *Oerskovia paurometabola* as potential model PAH-degraders. All of the isolates tested showed specific enzyme activities of meta cleavage of aromatic hydrocarbon degradation using catechol 1,2 dioxygenase. Biolog™ EcoPlates plates were used to evaluate the metabolism of selected isolates showing that the isolates could metabolize a wide range of organic substrates. These isolates have the potential to be used in bioaugmentation for aged-weathered soils with long-term contamination and nutrient limiting settings.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2018.02.004.

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