

Article

# Coal-Degrading Bacteria Display Characteristics Typical of Plant Growth Promoting Rhizobacteria

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**Abstract:** Coal mining produces large quantities of discard that is stockpiled in large dumps. This stockpiled material, termed coal discard, poses an environmental threat emphasising the need for appropriate bioremediation. Here, metagenomic analysis of the 16S rRNA from ten coal-degrading strains previously isolated from coal slurry from discard dumps and from the rhizosphere of diesel-contaminated sites was used to establish genetic relatedness to known plant growth-promoting (PGP) bacteria in the NCBI database. Measurement of indole and ammonium production and solubilisation of P and K were used to screen bacteria for PGP characteristics. BLAST analysis revealed  $\geq 99\%$  homology of six isolates with reference PGP strains of *Bacillus*, *Escherichia*, *Citrobacter*, *Serratia*, *Exiguobacterium* and *Microbacterium*, while two strains showed 94% and 91% homology with *Proteus*. The most competent PGP strains were *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b isolated from diesel-contaminated soil. In response to L-trp supplementation, the concentration of indolic compounds (measured as indole-3-acetic acid) increased. Production of ammonium and solubilisation of insoluble P by these strains was also apparent. Only *Serratia* strain ECCN 24b was capable of solubilising insoluble K. Production of indoles increased following exposure to increasing aliquots of coal discard, suggesting no negative effect of this material on indole production by these coal-degrading bacterial isolates and that these bacteria may indeed possess PGP characteristics.

**Keywords:** coal discard; diesel-contaminated soil; discard dumps; biodegradation; phylogeny; plant growth-promoting bacteria

## 1. Introduction

Coal is a hydrophobic, highly porous and heterogeneous sedimentary rock mined for its energy content. It is classified according to the degree of aromaticity and ranked as either lignite, (sub)-bituminous coal or anthracite [1,2]. Physically, it is a material with a hardness scale of up to 3 Mohs for anthracite and classified as naturally recalcitrant [3,4]. Chemically, it is a complex mixture of aromatic, heterocyclic and aliphatic carbonaceous constituents, hence the high calorific value. In South Africa, residual coal and other materials of very low energy content are stockpiled in discard dumps. These discard dumps are numerous and a major source and contributor of pollutants (e.g., particulate matter, heavy metals, acid leachate, etc.) to the surrounding environment including the atmosphere and water bodies and, as a consequence, threaten the environment, human health and quality of life [5,6]. Not surprisingly, there is an urgent need for intervention [7]. Although attempts to stabilise these dumps by cultivation of cover crops does occur once mining is complete [8], a major oversight in developing rehabilitation strategies has been the apparent omission of biotic factors that underpin

successful and sustainable revegetation. Recently, it was demonstrated that successful rehabilitation and vegetation of discard dumps can be achieved by exploiting the mutualism between coal-degrading microorganisms, mycorrhizal fungi and grasses [8].

Coal mining not only visibly disrupts the aesthetics of a landscape but also disrupts all of the soil components including soil horizons, structure, microorganisms and nutrient cycles that are crucial to sustaining a healthy ecosystem [9,10]. While a number of fungi show potential as rehabilitation biocatalysts and degrade coal into a soil-like humic-rich material [11–18], the only candidate enzymes identified and functionally characterised from coal-degrading fungi so far are laccase and esterase. Heterologous expression of *Fusarium oxysporum* laccase in *Pichia pastoris* was shown to depolymerise and liquefy solubilised brown coal with the release of humic and fulvic acids [18]. By comparison, the *Penicillium decumbens* P6 esterase depolymerised lignite resulting in increased formation of low-molecular mass humic acids with lower-percentage aromatic carbon but higher-percentage aliphatic carbon [19].

Bacterial conversion of coal, by comparison, has typically been to derive clean fuels and chemicals by developing environmentally sound energy-saving processes that do not cause secondary pollution [20,21]. While numerous studies on bacterial solubilisation and degradation of coal by different species of bacteria have been forthcoming, the underlying biochemical and molecular mechanisms involved are not yet fully understood [22,23]. Even so, numerous studies have revealed a role for bacteria in the degradative assimilation of petroleum hydrocarbons such as polynuclear aromatic hydrocarbons (PAHs), naphthalene, the monoaromatic hydrocarbons such as toluene and aliphatic hydrocarbons such as the *n*-alkanes [24,25]. These hydrocarbons are well represented in the naturally complex and highly aromatic coal structure and are also readily available in petroleum-contaminated soil environments [7]. Indeed, several recent extensive reviews on the subject have been published [26,27] and the potential for plant–bacteria partnerships in the remediation of hydrocarbon-contaminated soil has been proposed [28,29]. Bacterial conversion and utilisation of coal, though generally influenced by environmental conditions such as temperature and pH, are dependent mainly on the type of coal and microorganism used [29] and similar to fungal biodegradation, the mechanisms seem to involve solubilisation, depolymerisation and degradative assimilation [7]. Bio-solubilisation is by alkaline substances, chelators and/or surfactants, whereas depolymerisation and liquefaction likely entail oxidoreductases such as peroxidases and laccases, and hydrolases [30]. Under aerobic conditions, coal is oxidised by both biotic and abiotic processes to a weathered material rich in humic substances [31]. Under anaerobic conditions, a sequence of primary and secondary bacterial fermenters depolymerise and metabolise coal, providing a diverse range of short-chain organic acids and alcohols [32]. These low-molecular weight organic compounds serve as substrates for other consortia of microorganisms such as acetogens and methanogens [33–36]. In spite of many studies on the coal-degrading potential of bacteria, there remain limitations in phenotypic identification and the identification of other beneficial properties of these bacteria such as plant growth promotion. Modern molecular approaches, including analysis of PCR-amplified 16S rRNA gene sequences, have provided some insight into the identity, characterisation and phylogenetic classification of microorganisms capable of degrading and/or utilising coal [37–39]. To our knowledge, bacterial isolates from South African environments with coal-degrading capability have neither been extensively explored nor characterised genotypically.

An earlier bioprospecting study allowed for selected bacterial isolates with coal biodegradation potential to be characterised [40], but a more detailed biochemical and genetic analysis has yet to be described. Furthermore, screening of these coal-degrading bacteria for plant growth-promoting (PGP) characteristics has not previously been the subject of study. Defined as a class of microorganism that occupy the rhizosphere to promote plant growth, PGP bacteria are from diverse genera, display properties such as phytohormone production, siderophore and phosphate solubilisation activity, ACC deaminase activity and enhance plant productivity [41]. Typical uses are as inoculants for biostimulation, biocontrol and biofertilisation in agriculture and phytoremediation. In light of this

background, the present study sought to establish, first, the genetic relatedness of these isolates to the diverse array of PGP bacteria contained in the established NCBI database and, second, screen these coal-degrading strains for characteristics typically displayed by PGP bacteria. An ability to mineralise phosphate and potassium and to produce ammonium and indoles (including the phytohormone, indole-3-acetic acid) was the criteria used in this study to determine bacterial PGP potential.

## 2. Materials and Methods

### 2.1. Bacterial Strains

Bacterial strains used in this study were previously isolated from coal slurry obtained from coal discard dumps at coal mines in Emalahleni (26°0'0" S; 29°10'0" E), Mpumalanga Province, South Africa, and slurries of material from diesel-contaminated sites (at a depth of ~15 cm) collected either from soil at mechanical workshops in Makhanda (33°17'0" S; 26°31'0" E) or from old tractor engines at a farm in the Makana Local Municipality, Eastern Cape Province, South Africa (Table 1). Details of strain isolation, molecular characterisation, coal biodegrading activity and deposit into an international repository are recounted elsewhere [40].

**Table 1.** Bacterial strains with coal biodegrading activity isolated either from coal slurry (CS) or diesel-contaminated sites (DCS).

Source	Bacterial Isolates and Assigned Strain Number	Identity (%)	Microbial Culture Collection No.	GenBank Accession No.	Length (bp)
CS	<i>Citrobacter</i> strain ECCN 19b	99	MCC0033	KC700328	372
CS	<i>Escherichia</i> strain ECCN 25b	99	MCC0041	KC700329	382
CS	<i>Bacillus</i> strain ECCN 26b	99	MCC0062	KC700330	450
DCS	<i>Bacillus</i> strain ECCN 18b	98	MCC0034	KC620473	560
DCS	<i>Proteus</i> strain ECCN 20b	94	MCC0027	KC620475	553
DCS	<i>Exiguobacterium</i> strain ECCN 21b	99	MCC0016	KC620476	551
DCS	<i>Microbacterium</i> strain ECCN 22b	99	MCC0042	KC620477	543
DCS	<i>Proteus</i> strain ECCN 23b	91	MCC0022	KC620478	439
DCS	<i>Serratia</i> strain ECCN 24b	99	MCC0021	KC620474	525
DCS	<i>Bacillus</i> strain ECCN 41b	99	MCC0039	KC758162	392

### 2.2. Culture Conditions

Coal-degrading bacterial isolates, listed in Table 1, were resurrected from glycerol stock by culture in nutrient broth (NB; 5 g L<sup>-1</sup> peptone, 3 g L<sup>-1</sup> yeast extract and 8 g L<sup>-1</sup> NaCl) at 30 °C on a rotary shaker (120 rpm) for 72 h. Then, aliquots of NB were transferred to nutrient agar (NA; 5 g L<sup>-1</sup> peptone, 3 g L<sup>-1</sup> yeast extract and 8 g L<sup>-1</sup> NaCl and 15 g L<sup>-1</sup> agar) plates and incubated at 30 °C for 24 to 48 h to establish strain purity. Individual but distinct colonies were picked and inoculated into 40 mL of freshly prepared NB, grown at 30 °C, and maintained in log phase.

### 2.3. Extraction and PCR Amplification of Genomic DNA

Extraction of total DNA was achieved using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Target 16S rRNA regions were amplified using DreamTaq™ DNA polymerase (Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA) with the bacterial universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') as described by Lane et al. [42] and Turner et al. [43].

Sanger sequencing of the purified PCR products was carried out by Inqaba Biotechnical Industries (Pty) Ltd., Hatfield, South Africa using the PRISM™ Ready Reaction Dye Terminator Cycle sequencing kit using chain termination by the dideoxy-nucleotides methodology and electrophoresed on an ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Resultant chromatograms were analysed (Chromas version 2.6.6) as outlined in Technelysium [44], followed by pairwise alignment in BioEdit Sequence Alignment

Editor, version 7.0.5.3 [45], and the resultant gene sequences compared with those already deposited (<https://www.ncbi.nlm.nih.gov/>).

#### 2.4. Phylogenetic Analysis

Phylogenetic analysis using the 16S rRNA sequences of the nine isolates and 32 reference nucleotide sequences for PGP bacteria from the NCBI GenBank database was conducted. Multiple alignment of nucleotide sequences was achieved using CLUSTALW as implemented in the BioEdit Sequence Alignment Editor. A phylogenetic tree was constructed from the neighbour-joining method using MEGA6 (Molecular Evolutionary Genetics Analysis Version 6.0) after calculating distances via the Jukes–Cantor method [46]. Trees were bootstrapped using 1000 replications.

#### 2.5. Screening for Plant Growth Promoting Characteristics

##### 2.5.1. Ammonium Production

Production of ammonium ( $\text{NH}_4^+$ ) by the coal-degrading bacterial strains was determined using peptone water and Nessler's reagent ( $\text{HgI}_4\text{K}_2$ ) as indicator. Peptone water containing 10 g of peptone (Sigma-Aldrich, Millipore Sigma, St. Louis, MO) and 5 g of NaCl was prepared in 1 L distilled water and autoclaved at 121 °C and 1.5 kg  $\text{cm}^{-2}$  for 15 min. Nessler's reagent was prepared by suspending in 100 mL of distilled water, 10 g of  $\text{HgCl}_2$ , 7 g of KI and 16 g of NaOH. The bacterial inoculants were added to flasks containing 40 mL PW and incubated on a rotary shaker at 30 °C for 3 d. After incubation, 4 mL of each culture was harvested by centrifugation (3920×  $g$  for 3 min) and  $\text{NH}_4^+$  concentration of the supernatant quantified spectrophotometrically. For colorimetric assay of  $\text{NH}_4^+$ , to 2 mL aliquots of supernatant from each sample was added 0.2 mL of Nessler's reagent and colour development allowed to proceed for 5 min. Development of a brown colour was taken as a positive indicator of  $\text{NH}_4^+$ . For quantification, 1 mL was transferred to a cuvette and absorbance measured at 430 nm and results interpolated from a standard curve prepared using  $\text{NH}_4\text{Cl}$  [47].

##### 2.5.2. Indole-3-Acetic Acid Production

To determine the ability of the isolated bacterial strains to produce indole-3-acetic acid (IAA), NB was supplemented with L-trp and the concentration of indolic compounds produced by coal-degrading bacterial isolates determined using Salkowski's reagent [48–50].

Aliquots, equivalent to 40  $\mu\text{L}$  of bacterial suspension, were inoculated into NB supplemented with L-trp (at either 0, 0.1, 0.5 and 1  $\text{g L}^{-1}$ ) and incubated at 30 °C on a rotary shaker (120 rpm) for 72 h. After incubation, 4 mL of each culture was sedimented by centrifugation (3920×  $g$  for 3 min) and the cell-free supernatant used for colorimetric quantification of IAA which was carried out as follows: Two drops of 10 mM of orthophosphoric acid and 4 mL of Salkowski's reagent (50 mL 35% perchloric acid; and, 1.0 mL 0.5 M  $\text{FeCl}_3$  solution) were added to 2 mL cell-free supernatant, the mixture incubated at room temperature for 20 min and absorbance measured at 530 nm. The quantity of indolic compounds (expressed as IAA equivalents) was determined by interpolation from a standard curve of authentic IAA (Sigma-Aldrich) and background subtracted to account for any interference from the culture medium.

##### 2.5.3. Solubilisation of Insoluble Phosphate and Potassium

Phosphate and potassium solubilisation activity was initially determined using pour plates of either Pikovskayas agar (PVK) supplemented with tri-calcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ) or Aleksandrow medium (AM) containing  $\text{AlKO}_6\text{Si}_2$  as the respective substrates. Modified PVK agar was prepared using 0.5 g of yeast extract, 10 g of glucose, 5 g of  $\text{Ca}_3(\text{PO}_4)_2$ , 0.5 g of  $(\text{NH}_4)_2\text{SO}_4$ , 0.2 g of KCl, 0.1 g of  $\text{MgSO}_4$ , 0.0001 g of  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.0001 g of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 15 g of agar in a total volume of 1 L of distilled water [46]. For the solubilisation of potassium by coal-degrading bacteria, AM was prepared containing 0.5 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g of  $\text{CaCO}_3$ , 2 g of  $\text{AlKO}_6\text{Si}_2$ , 5 g of glucose, 0.005 g of

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 2 g of  $\text{Ca}_3(\text{PO}_4)_2$  and 20 g of agar in 1 L of distilled water. An aliquot (5  $\mu\text{L}$ ) of bacterial cell suspension was placed at 4 equidistant positions on either PVK or AM plates which were incubated at 30 °C for periods up to 14 d. After incubation,  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{AlKO}_6\text{Si}_2$  solubilising activity was determined by observation of clear zones around active bacterial colonies [51,52]. Phosphate and potassium solubilising activity was quantified after inoculation of either liquid PVK or AM, prepared as above but without agar, with equal quantities of bacterial suspension from cells in exponential phase.

Following incubation at 30 °C on a rotary shaker (120 rpm), cell suspensions were sedimented by centrifugation (3920 $\times$  g for 3 min) and the supernatant recovered for analysis. *Ortho*-phosphate concentration was determined spectrophotometrically using a Phosphate Test kit (Merck KGaA, Darmstadt, Germany) after 14 d while potassium concentration was measured after 9 d using sodium tetra-phenylborate as described by Pflaum and Howick [53]. The final concentration of phosphate and or potassium was determined by interpolation from standard curves prepared using *ortho*-phosphate and KCl, respectively.

### 2.6. Effect of Coal Discard on Production of IAA

Geologically weathered coal was sourced and prepared to yield particles of approximately 0.2–0.5 mm in diameter and sterilised by freeze-thawing using liquid nitrogen as described earlier by Olawale et al. [40]. Aliquots of dry sterilised coal (0–1 g) were added directly to seed cultures prepared from stationary phase cells (i.e.,  $\text{OD}_{600}$  0.4 after 3 d at 30 °C) of the specified coal-degrading strains. Nutrient broth (40 mL) supplemented with L-trp and incubated at 30 °C on a rotary shaker (120 rpm) for 3 d. Concentration of indolic compounds in solution was determined as described above after background subtraction to account for any coal-derived indoles.

### 2.7. Statistical Analysis

All data were computed using the statistical function in Sigma Plot version 11 (Systat Software Inc., San Jose, CA, USA). Where necessary, results were analysed by one-way analysis of variance and significant differences between measurements for each treatment were determined (Holm–Sidak method;  $p < 0.05$ ). Data are presented as the mean of at least three determinations  $\pm$  standard error (SE).

## 3. Results

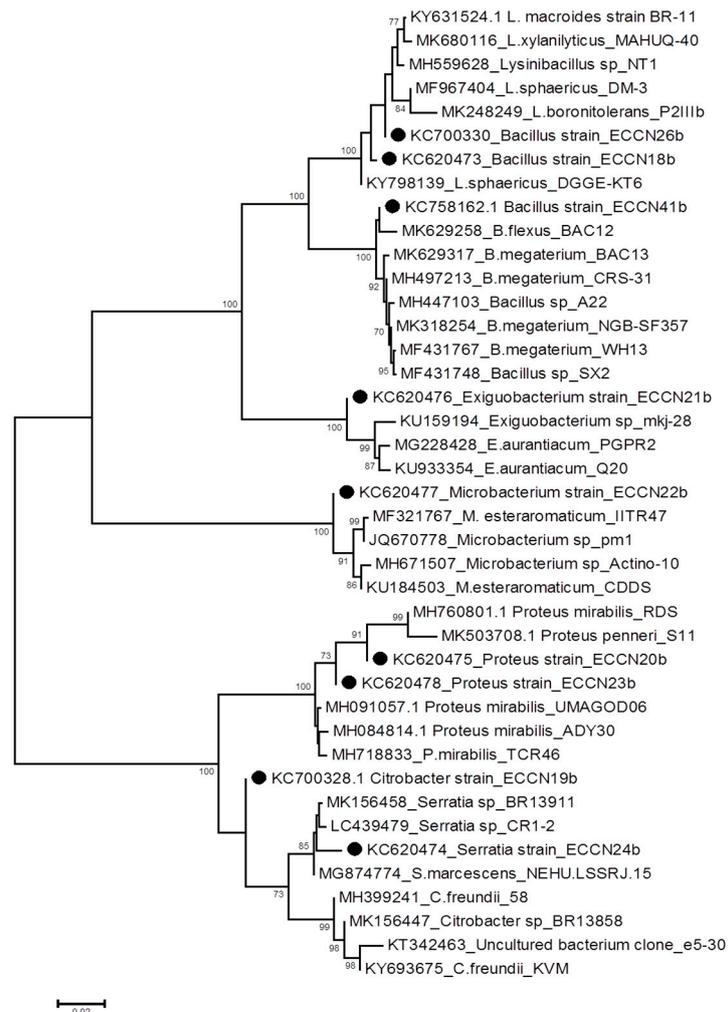
### 3.1. Bacterial Genomes and Their Identification

Partial 16S rRNA genomes of varying lengths ranging from 372 to 560 bp were amplified and sequenced from each of the resurrected coal-degrading bacterial strains previously isolated from coal slurry and diesel-contaminated soil. The ten strains represent seven genera from three families. The most abundant were Bacillaceae and Enterobacteriaceae, each with four strains and one Microbacteriaceae representative. Six isolates showed 99% sequence homology to reference strains that were confirmed as *Exiguobacterium* (KC620476), *Serratia* (KC620474), *Citrobacter* (KC700328), *Bacillus* (KC758162 and KC700330), *Escherichia* (KC700329) and *Microbacterium* (KC620477). One isolate, *Bacillus* (KC620473), showed 98% sequence homology, whereas KC620475 and KC620478 were 94% and 91% homologous to *Proteus*, respectively (Table 1). In this suite of previously identified coal-degrading bacterial strains, the most prevalent genus was *Bacillus*, accounting for 30%. Strains of *Proteus* comprising 20% of the isolates were the next most abundant and were from diesel-contaminated soil. Remaining strains were confirmed to belong to the genera *Escherichia*, *Citrobacter*, *Exiguobacterium*, *Serratia* and *Microbacterium*.

### 3.2. Nucleotide Sequence and Phylogenetic Analysis

The coal-degrading bacterial isolates were further characterised following phylogenetic analysis of sequence relatedness to PGP reference strain sequences within GenBank. Together with the selected 32 reference sequences from GenBank, our sequences revealed six distinct but associated clusters. Eight (8) sequences clustered individually with their respective PGP bacteria relatives, with virtually

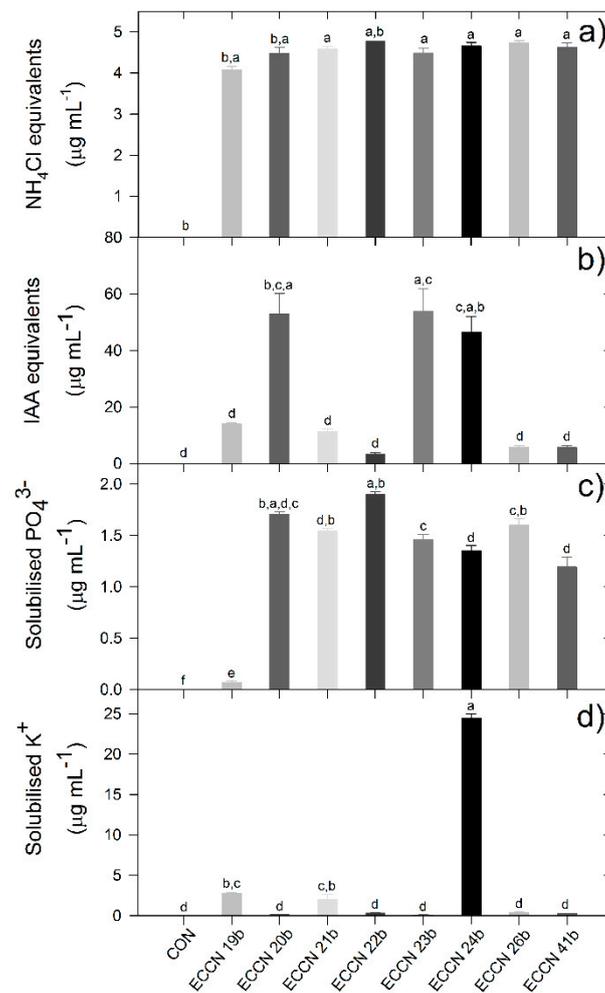
all showing 100% bootstrap support at the corresponding major nodes (Figure 1). However, strain ECCN 19b (KC700328) which was from a coal environment, was the stand-out within a major cluster consisting of the genera *Proteus*, *Serratia* and *Citrobacter*. Other sequences from strains within the same cluster (KC620475 and KC620478) formed a distinct cluster with *Proteus*, and KC620474 clustered distinctly within the *Serratia* clade (Figure 1). Although most clusters in the phylogenetic tree showed high bootstrap values, strain ECCN 25b (KC700329) was excluded from the constructed phylogenetic tree as it did not match closely with any of the available GenBank PGP reference sequences in relation to source.



**Figure 1.** Phylogenetic tree showing relation between coal-degrading bacterial isolates (●) and reference PGP strains contained in the NCBI GenBank database. GenBank accession numbers and the corresponding taxonomic annotations are shown to the right of the strains. Bootstrap values of  $\geq 70\%$  are shown at the nodes.

### 3.3. Plant Growth Promoting Characteristics

Production of ammonium and indolic compounds (measured as IAA equivalents) by the coal-degrading bacterial strains *Citrobacter* ECCN 19b (KC700328), *Proteus* ECCN 20b (KC620475), *Exiguobacterium* ECCN 21b (KC620476), *Microbacterium* ECCN 22b (KC620477), *Proteus* ECCN 23b (KC620478), *Serratia* ECCN 24b (KC620474), *Bacillus* ECCN 26b (KC700330) and *Bacillus* ECCN 41b (KC758162) and solubilisation of P and K were determined spectrophotometrically and the results are shown in Figure 2.



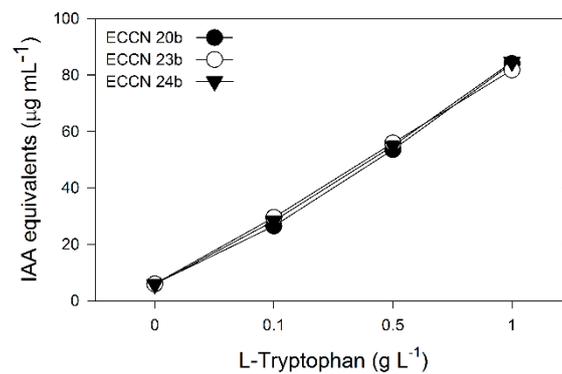
**Figure 2.** Screening of coal-degrading bacterial isolates for plant growth-promoting characteristics. Estimates of ammonium production (a), indole production (b), phosphate (c) and potassium solubilisation (d) by these bacterial isolates in stationary phase ( $OD_{600} = 0.4$  at  $30\text{ }^{\circ}\text{C}$ ) were determined spectrophotometrically. Results are expressed as the mean  $\pm$  S.E. Means denoted by a different letter indicate significant differences relative to a negative control ( $p < 0.05$ ).

Ammonium production appeared to be a common trait of the coal-degrading bacterial strains evaluated for PGP characteristics (Figure 2a). Furthermore, whereas all of the strains produced above background levels of indolic compounds (Figure 2b), *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b produced substantial quantities in response to L-trp ( $0.5\text{ g L}^{-1}$ ) supplementation (Figure 2b). Further, while it is accepted that mineral solubilisation can sometimes depend on the medium used [54], results from this study show that while phosphate solubilisation was low for these coal-degrading isolates, only one coal-degrading strain was unable to solubilise  $\text{Ca}_3(\text{PO}_4)_2$  (Figure 2c). By comparison, *Serratia* strain ECCN 24b, and to a much lesser extent *Citrobacter* strain ECCN 19b and *Exiguobacterium* strain ECCN 21b, were the only coal-degrading bacteria able to solubilise potassium from  $\text{AlK}_2\text{O}_6\text{Si}_2$  (Figure 2d).

Indole-3-acetic acid is a common, naturally occurring plant hormone of the auxin class, is derived from L-trp and is characteristically produced by many different PGP bacterial strains [55]. In an effort to gain further insight into the relationship between IAA production and the PGP potential of these coal-degrading bacterial strains, L-trp-activated IAA production by *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b was examined, firstly, in response to increasing concentrations of this indole

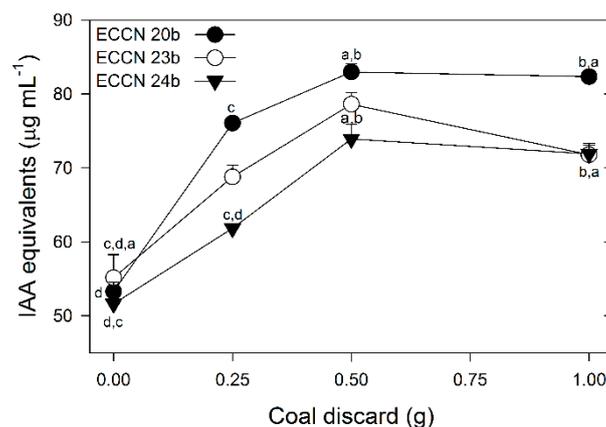
precursor and, secondly, following exposure of L-trp-supplemented cultures to increasing amounts of coal discard.

Results in Figure 3 clearly show that by increasing the concentration of L-trp supplied to cultures of *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b, production of indolic compounds including IAA, increased. Furthermore, these coal-degrading strains seemed equally competent in terms of indole production in response to activation by L-trp supplementation. Mean medium indolic compound concentration for the three strains increased from a mean minimum of  $5.91 \mu\text{g mL}^{-1}$  in the absence of L-trp to a mean maximum of  $83.49 \mu\text{g mL}^{-1}$  IAA (equivalents) in response to  $1 \text{ g L}^{-1}$  L-trp supplementation. The almost linear increase in medium indolic compound concentration ( $R^2 = 0.93$ ), concomitant with increasing concentration of L-trp supplementation, appears to support the operation of an L-trp-dependent pathway for indole/IAA production by these bacterial isolates.



**Figure 3.** Indolic compound formation by cultures of *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b in response to increasing L-trp concentration. Results are from three experiments and expressed as the mean  $\pm$  S.E.

In view of the already established coal-biodegrading property of these strains, it was of interest to examine the effect of coal discard on production of indolic compounds by cultures of *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b. Increasing quantities of coal discard were supplied to these isolates in liquid culture and the effect on L-trp-supplemented indolic compound accumulation is shown in Figure 4.



**Figure 4.** Effect of coal discard on formation of indolic compounds by cultures of *Proteus* ECCN 20b, *Proteus* ECCN 23b and *Serratia* ECCN 24b in response to L-trp supplementation. Results are from three experiments and expressed as the mean  $\pm$  S.E. Means denoted by a different letter indicate significant differences within treatments ( $p < 0.05$ ).

Typically, screening for production of IAA by putative PGP bacteria is carried out using an L-trp-supplemented medium [56,57]. Here, too, a L-trp-supplemented medium was used, and results show that in the presence of coal discard, production of indoles was enhanced for all three of the coal-degrading strains tested (Figure 4). In this experiment, coal was added to NB supplemented with L-trp at  $0.5 \text{ g L}^{-1}$ . In the absence of coal discard, formation of indoles averaged  $53.34 \mu\text{g mL}^{-1}$  IAA for the three strains tested, an observation that was similar to and corroborated initial screening studies (cf. Figure 2b). For strain ECCN 20b however, indole formation increased to 76.05, 82.96 and  $82.33 \mu\text{g mL}^{-1}$  IAA in response to addition of 0.25, 0.5 and 1 g of coal discard, respectively (Figure 4). A similar, albeit less dramatic, response was observed for strains ECCN 23b and ECCN 24b. Indeed, coal discard above 0.5 g appeared to slow accumulation of indoles in all bacterial isolates tested. Maximum IAA was thus achieved in response to 0.5 g-added coal discard, while competence for production of indolic compounds by these strains appeared to be ECCN 20b > ECCN 23b > ECCN 24b.

#### 4. Discussion

Molecular ecological studies involve separation of amplicons based on differences in DNA nucleotide sequences and most often the 16S rRNA gene, which can then be analysed from complex environments such as soil [58]. Databases thus generated yield invaluable information about indicator species [59]. To determine the phylogenetic relationship between coal-degrading bacteria isolated earlier [40] and the diverse array of PGP bacteria contained in the NCBI GenBank database, basic bioinformatics was used to determine the genetic relatedness of the 16S rRNA gene sequences while conventional biochemical assays were used to screen for putative PGP characteristics. Strains from seven genera of coal-degrading bacterial isolates utilised in the present study showed  $\geq 99\%$  homology with reference gene sequences of PGP bacteria contained in the NCBI GenBank database. A  $< 1\%$  deviation was attributed to the diversity of ecological niches from where the coal-degrading isolates and reference PGP strains were sourced.

Biochemical screening for traits typically associated with PGP showed that the most competent strains were those sourced from diesel-contaminated soil. These isolates were *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b. Interestingly, *Serratia* strain ECCN 24b, either alone or in consort with *Citrobacter* strain ECCN 19b and *Exiguobacterium* strain ECCN 21b, was previously shown to be most effective in coal bioconversion [40]. Furthermore, and in corroboration with results from other studies, *Serratia* strain ECCN 24b was able to solubilise insoluble phosphate [60,61] and potassium [62]. Recent studies have shown that the mineral-solubilising activity for promotion of plant growth is typically associated with the production of organic acids which, for strains of *Serratia* spp., appears to include gluconic and lactic acid [63].

In addition to the liberation of mineral nutrients, production of indolic compounds by *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b in L-trp-supplemented medium was substantial relative to the other coal-degrading bacterial isolates screened and increased linearly with an increase in L-trp concentration. Production of the plant hormone IAA and other indoles by bacteria is a long held and accepted view, in particular for microorganisms that inhabit subterranean plant surfaces [64–67]. Indeed, bacterial IAA biosynthesis can be either trp-dependent or independent [68] and results from the present study would seem to support the formation of indolic compounds by *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b via L-trp. Coal-degrading isolates that display a weak response to L-trp supplementation may either be non-responsive or produce indole by a trp-independent route. In higher plants like *Arabidopsis thaliana* and *Zea mays*, the major biosynthetic pathway to IAA is not via trp but seems to be from an earlier intermediate such as anthranilate [69]. Further, several studies have confirmed formation by bacteria of methyl anthranilate via chorismite in a single-step conversion of anthranilate to its methyl ester metabolite catalysed by an S-adenosyl-L-methionine (SAM)-dependent methyltransferase [70,71]. Whether a similar pathway operates in the coal-degrading strains that were unresponsive to L-trp supplementation in the present study is currently unknown.

Additionally, exposure of *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b to coal discard resulted in an apparent dose-dependent increase in the formation of indolic compounds. While indoles are a component of carbonised coal, e.g., coal tar and coke [72], and pyrolysates of Leonardite humic acids [73], they are not readily liberated from hard coal or coal discard, and in this study were undetectable in samples used to background correct for possible contribution to spectrophotometric quantitation using Salkowski's reagent.

In addition to the well-established PGP properties of *Serratia* spp. [74–77] and their ability to degrade the coal distillation product creosote [78], several promising PGP rhizobacterial strains of *Proteus* spp. have been isolated from the rhizosphere of rice [79–81], wild grass [82], a halophytic glasswort [83,84] and a nematocidal strain of *P. penneri* obtained from cow dung [85]. This genus was also relatively well represented (20%) among the coal-degrading isolates utilised in this study and strains ECCN 20b and ECCN 23b, isolated from diesel-contaminated soil. There are numerous reports of hydrocarbon-degrading strains of *Proteus* spp. from soils [86–88], whereas, to our knowledge, the strains described here represent the first reported *Proteus* isolates with PGP potential capable of degrading coal. While there is reported evidence for isolation of *Proteus* spp. from diverse soil environments including isolates with heavy metals tolerance [89–91] and xenobiotic-degrading capability [92,93], no coal-degrading strains have been documented. However, at least one report indicates that a strain isolated from two different ranks of coal was unable to degrade lignin [94].

## 5. Conclusions

Our goal in the present study was to determine the genetic relatedness of coal-degrading bacterial strains isolated from the rhizosphere of grasses growing on coal discard dumps and from diesel-contaminated sites to sequences of known PGP isolates contained in the NCBI GenBank database. We then determined the potential functionality of this relatedness by screening for PGP characteristics. Analysis of PCR-amplified 16S rRNA gene sequences from the seven genera of coal-degrading bacteria showed >99% homology to 32 sequences of known PGP strains from the NCBI GenBank database representing three families viz. four strains from each of the Bacillaceae and Enterobacteriaceae and one strain from the Microbacteriaceae. The Gram-negative isolates *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b, all from Enterobacteriaceae and isolated from diesel-contaminated soil, were shown to be competent for PGP characteristics using the biochemical parameters described in this study. Thus, while all strains were competent for ammonium and indole compound production, and solubilisation of insoluble phosphate, only *Proteus* strain ECCN 20b, *Proteus* strain ECCN 23b and *Serratia* strain ECCN 24b responded to the L-trp-supplemented medium by substantial accumulation of indolic compounds. Further, for these strains, indole compound accumulation in response to L-trp supplementation was linear with increasing L-trp concentration and not negatively affected in the presence of coal discard. Even so, it remains to be determined whether bioconversion of coal can occur with simultaneous production of indole compounds and in particular the auxin, IAA.

Further screening is therefore needed to expand our knowledge of the suite of PGP properties of the coal-degrading isolates. Using a combination of genome mining and metabolomics, possible target characteristics could include auxin and siderophore production, nitrogen fixation and potential phyto-protective mechanisms. It is also important to determine any direct effect of the isolated PGP coal-degrading bacteria on plant growth and development. A better understanding of the plant–bacteria relation may lead eventually to candidate strains for the development of a biological process with which to treat, stabilise and even rehabilitate disturbed soils.

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