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Isolation and Molecular Characterization of Soil Bacteria Capable of Degrading Chlorpyrifos and Diuron Pesticides

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Abstract The pesticides Chlorpyrifos and Diuron have been extensively used in sugarcane farming for several years in spite of their known harmful effects. The aim of this study was to isolate Chlorpyrifos and Diuron-degrading bacteria from exposed agricultural soil in the Nzoia River Drainage Basin and characterize the isolated bacteria by analyzing the 16S rRNA nucleotide sequence. The isolates could be potential candidates for use in bioremediation protocols. One soil isolate was found capable of degrading Chlorpyrifos and another was found capable of degrading Diuron. 16S rRNA gene sequences of the two isolates were deposited in GenBank and assigned the Accession Numbers MG517447 and MG517448 respectively. Sequence analysis of the two isolates using BLASTN and phylogenetic analysis revealed that the isolate capable of utilizing Chlorpyrifos as the sole carbon source was *Kosakonia oryzae* strain Ola 51, while the isolate capable of utilizing Diuron as the sole carbon source was *Pseudomonas aeruginosa* strain M-1**.** *Kosakonia oryzae* strain Ola 51 and *Pseudomonas aeruginosa* strain M-1 are thus potential candidates for use in bioremediation protocols for soils contaminated with Chlorpyrifos and Diuron, respectively.

Keywords: *Chlorpyrifos, Diuron, Kosakonia oryzae, Pseudomonas aeruginosa*

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1. Introduction

Chlorpyrifos [O, O-diethyl O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate] is a broad-spectrum, widely used organophosphate pesticide. It is used in protection of agricultural crops such as sugarcane, coffee, tea, cocoa, rice, wheat, potatoes, vegetables, bananas, citrus fruits and cotton. It is also used in the protection of domestic animals and built structures such as domestic houses and commercial establishments [1] and is one of the most widely used organophosphate pesticides worldwide [2]. Diuron [*N*-(3, 4-dichlorophenyl)-N, N-dimethylurea] is a substituted phenylurea herbicide used to control a wide variety of annual and perennial broadleaf and grassy weeds, as well as some mosses. In the Nzoia River Drainage Basin, both Chlorpyrifos and Diuron are used in sugarcane farming under the trade names Pyrinex and Diurex respectively [3]. Both pesticides are used repeatedly, depending on season and emergence of pests; leading to a significant amount of both pesticides ending up in soil and groundwater, thus affecting non-target organisms, as reported by some authors [4]. The heavy use of pesticides with known harmful effects and their persistence and mobility in the environment translate to a high risk of pesticide poisoning as discussed elsewhere [1,5]. Previous studies by Stork and Kaonga have detected residues of the pesticides in the environment at unacceptably high levels [6,7]. Bioremediation is the use of naturally occurring or deliberately introduced microbes to contaminated sites to break down or otherwise remove xenobiotic contaminants, in this case pesticides, from the environment. Bioremediation is potentially faster and cheaper than other methods of decontamination as it involves naturally occurring microorganisms breaking down contaminants through biodegradation. Bacterial degradation of Chlorpyrifos results in 3, 5, 6, trichloro-2-pyridinol (TCP) as a major metabolite [8], which is antimicrobial and toxic [9]. While some bacteria can only break Chlorpyrifos down to TCP, others have molecular and biochemical attributes that allow them to mineralize TCP at a rate higher than the rate of its formation, breaking it down completely into harmless organic matter and Carbon dioxide.

Microbial biodegradation of diuron under aerobic conditions yields 3,4-dichloroaniline (DCA); N-(3,4-dichlorophenyl)-N-methylurea (DCPMU); 3,4-dichlorophenylurea (DCPU), with DCA being the main metabolite, as previously studied [10]. Again, different microbes degrade Diuron to give different end products, with some being able to degrade the pesticide and its metabolites to CO_2. Identification of

bacteria capable of biodegrading pesticides efficiently through metagenomics is of vital importance in generating protocols for bioremediation using the bacteria as mediators of the process in situ or ex situ. In this study, pesticide- degrading bacteria were isolated from exposed agricultural soils in the Nzoia River Drainage Basin and identified using biochemical tests and 16 S rRNA analysis.

2. Materials and Methods

2.1. Sampling

The soil sampling sites were located in the Nzoia Sugar company nucleus estate, lying between longitudes 34°34'00"-34°51'30"E and latitudes 0°23'00"-0º37'30"N. Plots and sites to be used in sampling were identified using random stratified sampling technique. The sites selected were those with a long history of exposure to the pesticides (> 1 year). For Chlorpyrifos, soil was collected from termite mounds in and around sugarcane fields. Soil was collected from the surface of the termite mounds as well from deep within the mounds. For Diuron, soil was collected from the surface of fields, from a depth of 0-15cm. All the soil samples were placed in sterile Whirl Pak bags then placed in a cooler at 4°C for transport to the laboratory.

2.2. Reagents and Chemicals

Chlorpyrifos and Diuron analytical standards (≥99.5% purity) were obtained from Sigma-Aldrich Corporation, USA. Chlorpyrifos Mineral Salt Medium (MSM) was constituted as follows (in grams per liter of distilled water): $(NH_4)_2NO_3$, 1.0; Ca $(NO_3).2H_2O$, 0.04; MgSO4.7H2O, 0.1; KCl, 0.2; $FeSO_4 \cdot 7H_2O$, 0.001, $K_2HPO_4 \cdot 12H_2O$, 1.5; and KH_2PO_4, 4.8 and 1ml trace metal solution. The pH was brought to 7.0, as discussed by Rani [11]. The MSM was then autoclaved at 121°C for 15 minutes. Enrichment with Chlorpyrifos was done by aseptically dissolving Chlorpyrifos in a minimal volume of HPLC-grade methanol and adding it to the MSM solution at a concentration of 10mg/L. Diuron Mineral Salt Medium was constituted as follows (in grams per litre of distilled water): $NaNO_3$, 6.0; KH_2PO_4, 1.5; KCl, 0.5; $MgSO_4$.7H2O, 0.5; $FeSO_4$, 0.001; $ZnSO_4$, 0.001 and 1ml trace metal solution. The pH was brought to 6.8 as discussed by Dellamatrice [12]. Enrichment with Diuron was done by aseptically dissolving Diuron in a minimal volume of HPLC-grade methanol and adding it to the MSM solution at a concentration of 25 mg/L Tryptic Soy Broth Medium (TSB medium), Nutrient Agar, MacConkey Agar and 5% Agar-agar, all obtained from Sigma- Aldrich Corporation USA, were prepared according to the manufacturer's instructions.

2.3. Isolation of Pesticide-degrading Bacteria

2.3.1. Isolation of Chlorpyrifos-degrading bacteria

Isolation of Chlorpyrifos-degrading bacteria from soil samples was carried out by enrichment culture technique using a modification of the method described by Ifediegwu *et al*. [13]. Soil samples were crushed and air-dried, then passed through a 2-mm sieve. Ten 50ml bijou bottles were sterilized by autoclaving at 121°C for 15 minutes. Five of these bottles were filled with the MSM and five filled with TSB medium. 10 g soil samples were weighed and added to the solution in each bijou bottle. All the bottles were placed in a Wisecube rotational shaker at 120 revolutions per minute (rpm) for 48 hours at 37°C. At 12-hour intervals, one bottle each of CPF-enriched Mineral Salt Medium and Tryptic Soy Broth medium were removed from the shaker. From each bottle, a loop-full of solution was inoculated on nutrient agar and MacConkey agar using a sterile wire loop. The nutrient agar and MacConkey agar plates were incubated at 37°C for 24 hours. CPF mineral salt agar were prepared by first preparing a 5% Agar-agar solution and sterilizing it by autoclaving at 121°C for 15 minutes then aseptically adding CPF as the only carbon source (dissolved in minimal volume of HPLC-grade methanol) at a concentration of 10mg/L. All the bacteria which grew on the nutrient agar and MacConkey agar plates were transferred to CPF mineral salt agar using a sterile wire loop. CPF mineral salt agar plates were incubated for 7 days at 37°C until single colonies were observed. The plates were observed after every 24 hours for the appearance of single colonies. Soil from Kakamega Forest with no history of CPF exposure was used as a control. Isolates were stored at -80°C in tryptic soy broth with 15% glycerol for subsequent experiments.

2.3.2. Isolation of Diuron-degrading bacteria

Isolation of Diuron-degrading bacteria was carried out by enrichment culture technique using a modification of the method described by Ifediegwu *et al.* [13]. Soil samples were crushed and air-dried, then passed through a 2-mm sieve. Ten 50ml bijou bottles were sterilized by autoclaving at 121°C for 15 minutes. Five of these bottles were filled with the enriched MSM and five filled with TSB medium. 10 g soil samples were weighed and added to the solution in each bijou bottle. All the bottles were placed in a Wisecube rotational shaker at 120 revolutions per minute (rpm) for 48 hours at 37°C. At 12-hour intervals, one bottle each of diuron-enriched MSM and TSB medium were removed from the shaker. From each bottle, a loopful of solution was inoculated on nutrient agar and MacConkey agar. The nutrient agar and MacConkey agar plates were incubated at 37°C for 24 hours. Diuron mineral salt agar were prepared by first preparing a 5% Agar-agar solution and sterilizing it by autoclaving at 121°C for 15 minutes then aseptically adding Diuron as the only carbon source (dissolved in minimal volume of HPLC-grade methanol) at a concentration of 25mg/L. Single colonies which grew on the nutrient agar and MacConkey agar plates were transferred to Diuron mineral salt agar using a sterile wire loop. Diuron mineral salt agar plates were incubated for 14 days at 37°C until single colonies were observed. The plates were observed after every 24 hours for the appearance of single colonies. Soil from Kakamega Forest with no history of Diuron exposure was used as a control. Isolates were stored at -80°C in Tryptic Soy Broth with 15% glycerol for subsequent experiments.

2.4. Identification of pesticide-degrading isolates

Pesticide degrading isolates were identified using biochemical tests and 16 S rRNA analysis according to methods described by Rani *et al.* [11].

2.4.1. Biochemical Tests

Bacterial isolates were separately subjected to gram staining and subsequent biochemical tests. The tests performed were the Sulphur Indole Motility Medium (SIM) test, Nitrate test, Methyl Red/Voges-Proskauer test, Citrate, Oxidase, Urease, Catalase and Carbohydate fermentaton (Glucose, Sucrose and Lactose fermentation). The Sulphur Indole Motility Medium (SIM) is a differential medium used to carry out the Motility, Indole production and H_2S production tests. The Motility test tests a bacterium's ability to swim through the medium. The Indole production test tests production of Indole. The H_2S test tests the production of H_2S through reduction of Sulpur by the bacterium under study. The SIM test was done by inoculating SIM tubes using a pure, 18-hour-old colony of the bacterium under study with a single stab to the center of the medium to a depth of ½ inch. The medium was then incubated aerobically at 35°C for 24 hours. The medium was then observed for motility and H_2S production. A positive motility test is indicated by growth radiating from the line of inoculation, making the medium turbid. A negative motility test is indicated by growth confined to the stab line. A positive H_2S test is indicated by appearance of a black color in the medium along the line of inoculation. With a negative H_2S test, no black color is observed. After observing the results for motility and H_2S production, three drops of Kovacs reagent were added to the media to test for Indole production. A positive Indole test was indicated by a pink to red band at the top of the medium, while a negative test was indicated by a yellow color [14]. The nitrate test was done using Nitrate broth, which is a differential medium used to test a bacterium's ability to reduce nitrate (NO_3^-) to nitrite (NO_2^-). The medium was heavily inoculated using a pure, 18-hour-old colony of the bacterium under study and incubated aerobically at 35°C for 24 hours. One dropper-full of sulfanilic acid and one dropper-full of α-naphthylamine were added to the broth. At this point, a positive test was indicated by a red color, while a negative test was indicated by no color change. For further accuracy in this test, a small amount of Zinc was added. At this point, no color change indicated that no nitrate was formed, and this was a positive result. A red color at this point indicated a negative result [15]. The Methyl Red/ Voges-Proskauer test (MR/VP test) was used to determine the glucose fermentation pathway used by the bacterium under study. The MR/VP broth was inoculated using a pure, 18-hour-old colony of the bacterium under study and incubated aerobically at 35°C for 24 hours. 1 ml of the broth was transferred to a clean test tube. 0.6mL of 5% alpha naphthol was added, followed by 0.2 ml of 40% KOH. The test tube was then gently shaken and left undisturbed for 15 minutes. A positive test was indicated by a red color seen 15 minutes after addition of reagents [15]. The Citrate test was done using Simmons Citrate Agar. Using an 18- hour old pure colony of the bacterium under study, the Simmons Citrate Agar on the slant was gently inoculated on its surface. The slants were incubated for 24 hours. A positive result was indicated by a blue color, while a negative result was indicated by the medium remaining green [15]. The Oxidase test was performed by first soaking a filter paper in the substrate tetramethyl-p-phenylenediamine dihydrochloride and moistening the paper with distilled water. A pure colony of the bacterium under study, at the 18-hour growth stage, was smeared onto the filter paper using a sterile wooden swab. A positive test was indicated by a dark purple color developing in 10 seconds [15]. The Urease test was performed using a broth containing urea. The broth was inoculated using a pure, 18-hour-old colony of the bacterium under study and incubated aerobically at 35°C for 24 hours. A pink color indicated a positive result [15]. The Catalase test was done by adding 5 drops of 3% Hydrogen Peroxide (H_2O_2) to a test tube. A pure colony of bacterium under study was inoculated into the hydrogen peroxide using a sterile wooden applicator stick. A positive result was indicated by the immediate evolution of gas, shown by formation of gas bubbles [15]. The carbohydrate fermentation test was used to determine whether the bacterium under test was able to ferment a specific carbohydrate. The carbohydrates tested were glucose, lactose and sucrose. Phenol red glucose, Phenol red lactose and phenol red sucrose broths were used for glucose, lactose and sucrose, respectively. In each test, the broth was placed in a test tube, with a Durham tube inserted to detect gas production. The broth was inoculated with a loopful of a pure colony of the bacterium under study. The tubes were incubated at 35°C for 24 hours. A positive result was indicated by a yellow color and/ or a bubble appearing in the inverted Durham tube. The yellow color indicated fermentation of the respective carbohydrate to give an acid. The bubble of gas seen in the inverted Durham tube indicated fermentation which produced a gas [14].

2.4.2. Bacterial DNA extraction, PCR of 16SrRNA gene, Extraction and Purification

Genomic DNA from the bacterial isolates was extracted using Qiagen microbial DNA extraction kit following the manufacturer's protocol. The universal primers 27F (5'AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' TACGGCTACCTTGTTACGACTT 3') were used in PCR amplification of the extracted genomic DNA. The amplification step of PCR was carried out in reaction solutions containing the following: 0.5 µL of 27F primer (200ng/µL), 0.5 µL of 1492R primer (200ng/µl), 2.5 µL10X PCR reaction buffer, (100mMTris-HCl, 500 mM KCl, pH 8.3), 1.5 µL 25 mM$MgCl_2$ solution, 4.0 µL 1.25 mM, dNTPs, 0.1 µL AmpliTaq Gold DNA polymerase enzyme and 2 µL of DNA as the template. The reaction volumes were made up to 25 µL using sterile ultrapure water. The profile of thermal cycling consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation (for 1 min at 94°C), annealing for 1 min at 57°C, and extension for 2 min at 72°C, followed by a final extension for 8 min at 72°. As a safeguard against false positives which may arise from reagent contamination, negative controls were included. Amplicons were separated on 1.5% agarose gels in

1XTBE buffer at 10V cm^{-1} for 30 minutes. After this, the amplicons were stained with Ethidium bromide and observed using a BioRad UV transilluminator. The gel was photographed and the bands in it were located using a UV lamp. The bands were cut out and placed in a 2mLeppendorf tube. The PCR fragments were then extracted from the gel using Qiagen Gel extraction kit following the manufacturer's protocol.

2.4.3. Phylogenetic analysis of the 16SrRNA gene

The 16SrRNA gene sequence reads from the sequencer were edited using ChromasLite (http://www.technelysium.com.au/chromas_lite.html) to remove unknown bases. Consensus sequences were generated from sequence fragments using BioEdit version (version 7) [16]. A BLASTN search was done on the GenBank database in NCBI. One closely related homologous sequence of isolate 7, and three closely related ones of the isolate 21 were uploaded from the NCBI GenBank. Alignment was done using ClustalW [17] for phylogenetic comparison. The evolutionary history was inferred using the Neighbor-Joining method as described by Saitou and Nei [18]. The bootstrap consensus tree inferred from 1000 replicates [19] was taken to represent the evolutionary history of the taxa analysed [19]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches [19]. The evolutionary distances were computed using the Jukes-Cantor method as described by Jukes and Cantor [20] and are in the units of the number of base substitutions per site. Codon positions included were 1^{st}, 2^{nd}, 3^{rd} and noncoding. All positions containing gaps and missing data were eliminated.

3. Results

3.1. Isolation of Pesticide-degrading Bacteria

Thirty-nine bacterial isolates were obtained from sample soils and subsequently tested for their ability to utilize each of the pesticides. Two bacterial isolates were found capable of pesticide degradation. The isolate labeled 7 successfully utilized Chlorpyrifos as the sole source of carbon, while the isolate labeled 21 successfully utilized Diuron as the sole source of carbon. Control soil from Kakamega forest, which had no history of pesticide exposure, had no bacteria capable of growing on either pesticide mineral salt agar.

3.2. Biochemical Tests

The two bacterial isolates, 7 and 21, were observed growing on nutrient agar plates at the 18-hour growth stage for physical and morphological attributes. Table 1 shows the results for gram staining and biochemical tests.

Table 1. Biochemical characteristics of Chlorpyrifos- degrading (Isolate 7) and Diuron-degrading (Isolate 21) bacteria isolated from soil of sugarcane fields in the Nzoia River Drainage Basin, Kenya

Test/ Observation	Isolate 7	Isolate 21
Physical Characteristics	Color-Whitish-cream ; Margin-Entire; Texture-smooth; Elevation- Flat	Color-Greenish; Margin-Entire; Texture-soft; Elevation- Flat
Morphological Characteristics	Circular, Convex	Circular
Gram's Reaction	-	-
Growth on MacConkey Agar	-	-
Voges-Proskauer	+	-
Methyl Red	-	-
Motility	-	+
Indole	-	+
Citrate	+	+
Catalase	+	+
Oxidase	-	+
Nitrate	-	+
H_2S	-	-
Urease	-	-
Lactose	+	-
Glucose fermentation	+	-
Sucrose	+	-

3.3. Genomic DNA Isolation and PCR Analysis

Genomic DNA was successfully extracted for all the isolates and PCR amplification resulted in about 1400 base pair bands as shown in Figure 1

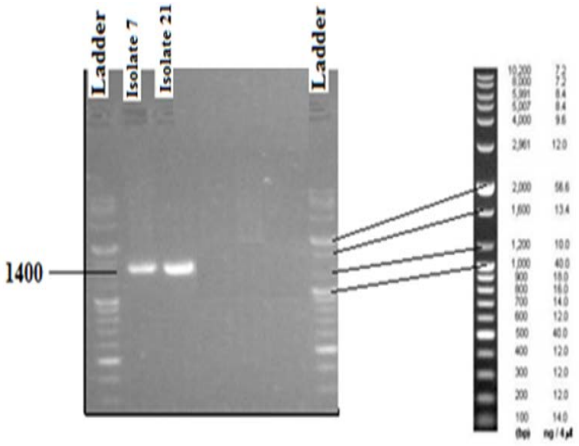

Figure 1. Gel electrophoresis of 16S rRNA PCR products observed on 1% Agarose gel

3.4. Phylogenetic Data Analysis

Quality control (Cleaning) was performed on sequence reads and consensus sequences were generated in Bioedit 7. BLAST search (BLASTn) was then performed in the GenBank database. The results are shown in Table 2

Table 2. Identities of pesticide-degrading bacterial isolates

Sample Label	NCBI Accession	Species name
Isolate 7	MG517447	Kosakonia oryzae
Isolate 21	MG517448	Pseudomonas aeruginosa

Phylogenetic tree in Figure 2 revealed two main clusters. Cluster A contained isolate 21 and NCBI GenBank accessions KF977857.1, KF670598.1 and KY885165.1, all of them identified as *Pseudomonas aeruginosa* while cluster B contained isolate 7 and NCBI GenBank accession CP014007.1 named as *Kosakonia oryzae*.

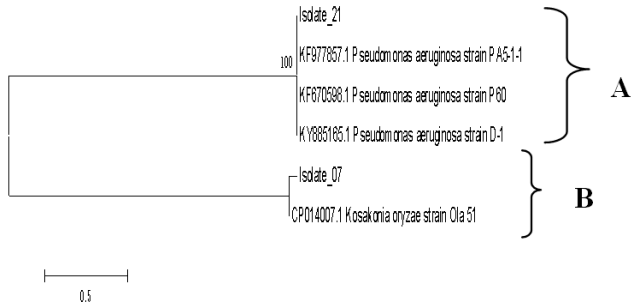

Figure 2. Evolutionary relationships between isolates and NCBI accessions

Genetic distance analysis indicated that members of cluster A had overall genetic distance of 0.0000. Even the distance between isolate 21 and all the three NCBI isolates individually had 0.0000 (Table 3). The genetic distance between isolate 7 and the NCBI hit with the accession number CP014007.1 was 0.0197. This zero or negligible genetic distance confirms that isolate 07 *Kosakonia oryzae* while isolate 21 is *Pseudomonas aeruginosa*.

Table 3. Estimates of Evolutionary Divergence between isolates and NCBI reference Sequences

Isolate	NCBI GenBank reference strain	Genetic distances
Isolate 7	CP014007.1 *Kosakonia oryzae* strain Ola 51	0.0197
Isolate 21	KF977857.1 Pseudomonas aeruginosa strain PA5-1-1	0.0000
Isolate 21	KF670598.1 Pseudomonas aeruginosa strain P60	0.0000
Isolate 21	KY885165.1 Pseudomonas aeruginosa strain D-1	0.0000

4. Discussion

The Motility test was used to determine if the bacterial isolate being tested possessed flagella. The Catalase test was used to determine whether the isolate involved produced the enzyme catalase, which breaks down hydrogen peroxide to oxygen and water [14] The Voges-Proskauer test and Methyl Red test were done in conjunction to determine the glucose fermentation pathway used by the bacterial isolate involved. The Voges-Proskauer was used to detect the presence of acetoin produced during fermentation of glucose to 2, 3 butanediol, with a positive test indicating that the bacterial isolate involved fermented glucose to 2, 3 butanediol. The Methyl Red test was used to determine the presence of organic acids, with a positive test indicating that the bacterial isolate involved fermented glucose to organic acids [15]. The Indole Test was used to determine the presence of the enzyme tryptophanase in the isolate under test [15]. This enzyme converts the amino acid tryptophan to indole, with a positive test indicating that the bacterial isolate involved was able to break down tryptophan [14]. The Citrate test was used to determine whether the bacterial isolate involved could utilize citrate as a carbon source, breaking it down through a series of steps into pyruvic acid and CO_2 [14]. The Oxidase test was used to determine the presence of cytochrome oxidase enzyme in the isolate, an enzyme in the terminal step of the electron transport chain [15]. The Nitrate test was used to determine whether the bacterial isolate involved could reduce nitrate to nitrite using nitrate reductase enzyme. The H_2S test was used to determine whether the bacterial isolate involved could break down Sulphur in the amino acid cysteine to H_2S [15]. The Urease test was used to assess the bacterial isolate's ability to break down urea using urease enzyme [14]. Glucose, Lactose and Sucrose fermentation for the bacterial isolates were tested using Phenol Red with the respective carbohydrate to determine if acidic, gaseous or both acidic and gaseous end products were produced from fermentation [15]. As described by Li [21], *Kosakonia oryzae* is a gram negative, rod-shaped non-spore forming nitrogen fixing bacterial species isolated from surface sterilized roots belonging to the wild rice species freely growing in China. It was found to be positive for acetoin production (Voges-Proskauer test) while negative for indole production; positive for β-galactosidase and arginine dihydrolase while negative for lysine decarboxylase; positive for oxidation of arabinose, cellobiose, citrate, fructose, galactose, gluconate, glucose, glycerol, lactose, malate, maltose, mannitol, mannose, sorbitol, sucrose and trehalose in previous studies by Peng *et al.* [22] and Brady *et al.* [23]. Though this bacterium is aerobic, it was known to reduce N_2 to NH_3 at low pO_2 concentration [21]. Most of these characteristics were found true with isolate 7 (Table 1) confirming that isolate 7 was indeed *Kosakonia oryzae*. Phylogenetic analysis of the isolate 07 as well showed that its 16S rRNA was closely related to *Kosakonia oryzae* strain Ola 51^T. The highest 16S rRNA gene sequence similarity (following comparison of 1436 bp of the 16S rRNA gene sequence) of isolate 07 was found with *Kosakonia oryzae* strain Ola 51^T (95 % similarity). Genetic distance between isolate 7 and *Kosakonia oryzae* strain Ola 51^T was very small (0.0197). This therefore further confirms that isolate 7 is *Kosakonia oryzae* but a different strain. To the best of our knowledge, *Kosakonia oryzae* has not been previously found to be capable of degrading Chlorpyrifos or other organophosphate pesticides. A widely distributed organophosphate-degrading gene (opd) was identified in geographically and biologically different species in studies by Richins *et al.* [24] and Wang *et al.* [25] but *Kosakonia oryzae* was not one of them. To date, there is no single

study that has found the existence of opd gene in this species. This might suggest that *Kosakonia oryzae* found in this study had obtained Chlorpyrifos degrading genes from other opd positive bacteria found in soil, since the gene has been found in other soil bacteria like those discussed by Singh [26] which utilize organophosphates as major carbon sources. Isolate 21 was able to grow in the media containing Diuron pesticide as a sole carbon source. This indicated that it has the ability to degrade this environmental pollutant. Widehem *et al.* [27] previously isolated microorganisms able to degrade Diuron though this degradation resulted in the formation of the harmful metabolite 3, 4-dichloroaniline which was further observed to be degraded by the fungi studied by Widehem *et al.* [27] and *Arthrobacter sp*. bacteria studied by Tixier *et al.* [28]. *Sphingobacterium sp*., an isoproturon-degrading bacterium, was also found to degrade Diuron in a study by Sorensen [29]. *Acinetobacter sp*. was also observed by Roque *et al.* [30] to mineralize Diuron when added to mineral media containing Diuron as the only carbon source. In an attempt to identify and characterize isolate 21, various biochemical tests were performed and the results authenticated by phylogenetic analysis. Biochemical tests showed that isolate 21 was gram negative rods, motile, catalase and oxidase positive and can also utilize nitrate in respiration. These characteristics point to the *Pseudomonas spp*. as studied by Woo *et al.* [31]. Blasting at NCBI GenBank returned three *Pseudomonas aeruginosa* strains with 92% identity (Table 3). Isolate 21 formed a cluster with these three *Pseudomonas aeruginosa* strains with equal branch length (Figure 2). Genetic distance analysis of the 16S rRNA showed that genetic distances between isolate 21 and all the three strains were zero (Table 3). These analyses therefore confirmed that isolate 21 was *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* has been previously reported by Dwivedi *et al.* [32] to degrade Isoproturon, which is a phenylurea herbicide like Diuron. Perennial use of Chlorpyrifos and Diuron resulted in repeated exposure of soil bacteria to the pesticides over long periods of time. This exposure resulted in the bacteria developing the capability to utilize the pesticides as sources of carbon, evidenced by their ability to visibly grow on media with the respective pesticides as the only sources of carbon, a trait not seen with soil bacteria from unexposed soil. This capability likely arose through genetic adaptation, since biodegradation processes of both pesticides have been shown to be catalyzed by enzymes encoded by genes as described in studies elsewhere [33,34]. Analysis using 16S rRNA gene was chosen due to the large size of the gene and due to the fact that it is generally highly conserved, which makes it suitable for identifying the genera of bacteria under study.

5. Conclusion

The results indicate that *Kosakonia oryzae* (Isolate 7) and *Pseudomonas aeruginosa* (Isolate 21) are potential candidates for use in soil bioremediation of Chlorpyrifos and Diuron respectively due to their ability to break down the pesticides through biodegradation. Suggested issues for further investigation include monitoring the rate and extent of biodegradation of each bacterial isolate on its respective pesticide, and investigation of biodegradation of the pesticides by these isolates under field conditions.

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Statement of Competing Interests

The authors declare no competing interests.

List of Abbreviations

MSM- Mineral Salt Medium
TSB- Tryptic Soy Broth
CPF- Chlorpyrifos

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