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Aerobic Degradation of 2,4-dichlorophenoxyacetic Acid and Other Chlorophenols by *Pseudomonas* Strains Indigenous to Contaminated Soil in South Africa: Growth Kinetics and Degradation Pathway[1]

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Abstract— Three indigenous pseudomonads, *Pseudomonas putida* DLL-E4, *Pseudomonas reactans* and *Pseudomonas fluorescens,* were isolated from chlorophenol-contaminated soil samples collected from a sawmill located in Durban (South Africa). The obtained isolates were tested for their ability to degrade chlorophenolic compounds: 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol (2,4-DCP) and 2,4,6-trichlorophenol (2,4,6-TCP) in batch cultures. The isolates were found to effectively degrade up to 99.5, 98.4 and 94.0% with a degradation rate in the range of 0.67–0.99 (2,4-D), 0.57–0.93 (2,4-DCP) and 0.30–0.39 (2,4,6-TCP) mgL^{-1} day^{-1} for 2,4-D; 2,4-DCP and 2,4,6-TCP, respectively. The degradation kinetics model revealed that these organisms could tolerate up to 600 mg/L of 2,4-DCP. Catechol 2,3-dioxygenase activity detected in the crude cell lysates of *P. putida* DLL-E4 and *P. reactans* was 21.9- and 37.6-fold higher than catechol 1,2-dioxygenase activity assayed, suggesting a meta-pathway for chlorophenol degradation by these organisms. This is also supported by the generally high expression of *C23O* gene (involved in meta-pathway) relative to *tfdC* gene (involved in ortho-pathway) expression. Results of this study will be helpful in the exploitation of these organisms and/or their enzymes in bioremediation strategies for chlorophenol-polluted environment.

Keywords: chlorophenols, *Pseudomonas* spp., 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP)

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Advances in agricultural practices have led to the production of new recalcitrant organic compounds such as chlorophenolic compounds which are widely used in many petroleum refineries, pharmaceutical industries, as well as in the production of herbicides, pesticides and preservatives of wood and textiles [1]. In addition to industrial production, chlorophenols are produced from naturally found phenols through chlorine bleaching of wood pulp in the paper industry and chlorination of domestic water supplies and swimming pools [2]. Due to the many uses and presence of chlorophenols in the environment, humans and animals are easily exposed to these compounds with consequent and immediate ill effects [3]. Currently, traditional methods of incineration and adsorption by solid phase are employed to get rid of these harmful compounds from the environment [4]. However, these methods are costly, labor intensive and result in the production of harmful secondary pollutants [1]. Thus, there is a need for an alternative method to ensure effective and efficient removal of various chlorophenolic compounds. Bioremediation approach, utilizing bacterial isolates, for the removal of chlorophenols from polluted environment is a promising alternative that has shown great results [1, 5].

Microorganisms have been isolated that degrade 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol (2,4-DCP) and 2,4,6-trichlorophenol (2,4,6-TCP) [6] and the diversity of microorganisms present in 2,4-DCP-contaminated environments has been examined to some extent using biochemical and DNA-based molecular techniques [7]. One approach to obtaining such information is to examine the expression of key functional genes involved in 2,4-DCP degradation and a direct correlation has been observed between the transcripts of *tfdA, tfdC* and *C23O* mRNAs and microbial degradative activity in soil [8]. Based on pure culture studies, it is established that aerobic degradation of chloroaromatics generally proceeds via chlorocatechols as central intermediates [7]. These chlorocatechols are usually further metabolized via a modified ortho-cleavage pathway as in

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Cupriavidus necator JMP134 [9], *Azotobacter chroococcum* [10], *Pseudomonas putida* MT2 [11] and *Pseudomonas arvilla* C-1 [12] that involves intradiol cleavage of the aromatic ring by a chlorocatechol 1,2-dioxygenase. However, bacteria including, *Pseudomonas pickettii* PKO1, *Azotobacter* sp. GP1, *Comamonas testosteroni* JH5 and BRC60, *Sphingomonas putida* GJ31 and *Sphingomonas* sp. BN6 could transform chlorophenols via meta-cleavage [13–16].

Despite various reports on bacterial degradation of chlorophenols, there is paucity of research incorporating microorganisms indigenous to contaminated sites in Africa. The aim of the study was to assess the ability of some indigenous *Pseudomonas* spp. isolated from chlorophenol-contaminated soil samples collected from a sawmill located in Durban (South Africa) for the degradation of 2,4-D; 2,4-DCP and 2,4,6-TCP in batch cultures. In addition, during 2,4-DCP degradation, the enzyme activity and expression of catechol 1,2- and catechol 2,3-dioxygenase genes encoding the enzymes involved in intradiol and extradiol cleavage of catechol, respectively, were examined. This could provide an insight into the relevance of the ortho- and meta-cleavage pathways during chloroaromatic degradation in contaminated environment and their implications in bioremediation strategies.

MATERIALS AND METHODS

Bacterial strains, culture conditions and chemicals. The bacterial isolates: *Pseudomonas putida* DLL-E4, *Pseudomonas reactans* and *Pseudomonas fluorescens* were enriched from chlorophenol-polluted soil samples collected from a sawmill in Durban area of South Africa via culture enrichment technique [17] using 2,4-dichlorophenol as the sole carbon source. Cultures of the isolated organisms were stored as glycerol stocks in the culture collection of the Department of Microbiology, University of KwaZulu-Natal (Westville Campus), Durban, South Africa. All the isolates were grown on nutrient broth (Merck, Germany) for inoculum preparation. 2,4-D; 2,4-DCP; 2,4,6-TCP and catechol were purchased from Sigma-Aldrich (USA). All other chemicals used were of analytical grade. All experiments were performed in triplicate and the average values were indicated throughout the manuscript unless otherwise stated.

Bacterial identification and phylogenetic analysis. Identification of the bacterial isolates was conducted via PCR amplification, sequencing and analysis of 16S rRNA gene sequences using previously described methods [18]. The evolutionary relationship of the isolates was inferred by comparing the 16S rRNA gene sequences of the bacterial isolates with those of other *Pseudomonas* spp. in the GenBank database (http://www.ncbi.nlm.nih.gov) previously reported to degrade 2,4-DCP and a phylogenic tree constructed as previously described [19].

Biodegradation of chlorophenols. The biodegradation of 2,4-D; 2,4-DCP or 2,4,6-TCP was performed in minimal salt medium (MSM) comprising of (g/L): K_2HPO_4, 4.36; NaH_2PO_4, 3.45; NH_4Cl, 1.0 and $MgSO_4 \cdot 6H_2O$, 0.912 supplemented with trace solution (1 mL/L) consisting of (g/L): $CaCl_2 \cdot 2H_2O$, 4.77; $FeSO_4 \cdot 7H_2O$, 0.37; $MnCl_2$, 0.10 and $NaMoO_4 \cdot 2H_2O$, 0.002. The substrates; 2,4-D; 2,4-DCP or 2,4,6-TCP were added separately as sole carbon source at a final concentration of 40 ppm [20]. Ten mL of 18 h grown bacterial cultures, standardized to OD_{600} of 1.0 according to McFarland standards [21], were added to 250 mL Erlenmeyer flasks containing 90 mL MSM and 40 ppm chlrophenols. One mL aliquots were removed aseptically from each flask at 24 h intervals over a 6 day period and measured for disappearance of 2,4-D; 2,4-DCP and 2,4,6-TCP at 295 nm, 285 nm, 311 nm, respectively [5, 22]. MSM containing no bacteria were used as negative controls. The standard curves for each compound were derived by plotting the absorbance at the respective wavelengths versus known concentrations of the respective chlorophenols. The degradation rate constant (k_1) of each compound was determined using Eq. (1) [23].

$$\ln(C_t/C_0) = -k_1 t, \quad (1)$$

where C_0 and C_t are the concentrations of 2,4-DCP at time 0 and t, respectively.

Determination of 2,4-DCP inhibition constant. The inhibition constant of 2,4-DCP on the growth of the isolates was determined according to Kargi and Eker [24], with some modifications. Ten mL of standardized culture (OD_{600} of 1.0) was added to 90 mL of MSM containing glucose (1 g/L). 2,4-DCP was added as sole carbon source at initial concentrations of 200, 400, 600, 800, 1000 or 1200 mg/L. The flasks were incubated at 30°C with continuous shaking at 150 rpm for 6 days. The concentration of 2,4-DCP was determined in the flasks daily and the substrate inhibition constant (K_{si}; mg/L) was calculated by fitting the data in Eq. (2) [24].

$$R_s = R_m/((1 + K_s/S)(1 + S/K_{si})), \quad (2)$$

where R_s and R_m are the actual and maximum initial rates of 2,4-DCP degradation (mg L^{-1} h^{-1}); S is the initial 2,4-DCP concentration (mg/L) and K_s is the saturation constant (mg/L).

Preparation of crude extracts and enzyme assays. For the preparation of crude extracts, the cells were grown for 18 h in nutrient broth and then exposed to phenol (600 ppm) for 2 h at 30°C for enzyme induction. The cells were harvested by centrifugation (10 min at 10000 × *g*) and resuspended in 50 mM Tris-sulfate buffer (pH 7.4) containing 1 mM β-mercaptoethanol and 1 mM EDTA (TEM buffer). Cells were washed once in TEM buffer (10 min at 10000 × *g*) and lysed on ice using sonic Ruptor 400 ultrasonicator (OMNI International, USA) (6 cycles each for 15 s

Table 1. Primers used in this study for quantification of *tfdC* and *C23O* gene expression

Primer	Sequence (5'–3')	Reference
CCDb F	GTGTGGCA(CT)TCGACGCCGGA(CT)	[7]
CCDe R	CCGCC(CT)TCGAAGTAGTA(CT)TGGGT	[7]
C230 F	AAGAGGCATGGGGGCGCACCGGTTCGATCA	[7]
C230 R	AACAAA(AGT)GCGC(GC)GTCATGCGG	[7]
16S F	CCTACGGGAGGCAGCAG	[26]
16S R	ATTCCGCGGCTGGCA	[26]

Table 2. Peak growth (OD_{600}) of isolates in the presence of chlorophenols

Chlorophenol	Microorganism		
	P. putida DLL-E4	*P. reactans*	*P. fluorescens*
2,4-D	0.282	0.244	0.240
2,4-DCP	0.264	0.252	0.197
2,4,6-TCP	0.268	0.300	0.228

pulse). Crude extract obtained by centrifugation (30 min at 10000 ×*g*) was used for enzyme activity assay. The presence of catechol 1,2-dioxygenase or catechol 2,3-dioxygenase was measured based on the appearance of ortho-cleavage product *cis,cis*-muconic acid or meta-cleavage product 2-hydroxymuconic semialdehyde, respectively, in the reaction using catechol as the substrate. The reaction mixture was prepared in a quartz cuvette containing 1 mL of 50 mM Tris-HCl buffer (pH 8.0); 0.35 mL of distilled water; 0.05 mL of 100 mM 2-mercaptoethanol, and 0.05 mL of cell-free extract. Catechol (0.05 mL, 1 mM) was added to initiate the reaction and the OD_{260} and OD_{375} was read continuously for 5 min for the appearance of *cis,cis*-muconic acid and 2-hydroxymuconic semialdehyde, respectively. The absorbance was converted to the molar concentration of products by using molar extinction coefficients, ε_{260} of 16800 L mol^{-1} cm^{-1} for *cis,cis*-muconic acid and ε_{375} of 14700 L mol^{-1} cm^{-1} for 2-hydroxymuconic semialdehyde. One unit of enzyme activity was defined as the amount of enzyme resulting in the production of one micromole product per min under standard assay conditions [25].

Quantification of *tfdC* and *C230* gene expression via real time PCR. A loopful of 18 h grown cells (OD_{600} of 0.1) was added to fresh 100 mL nutrient broth; culture was grown at 30°C till the OD_{600} reach 0.7 and further exposed to 600 mg/L 2,4-DCP for 2 h. The aliquots of 5 mL cultures were removed after 0, 5, 10, 20, 40, 60, 100 and 200 min, and pellets were collected by centrifugation as described above. Total RNA was isolated from the pellets using the trizol method according to manufacturer's instructions (Life technologies, Thermo Fisher Scientific Inc., USA) and converted to cDNA using ImProm-II reverse transcription kit (Promega, USA). The cDNA was stored at –70°C until further use for RT-PCR experiments. For relative quantification, *tfdC* and *C230* genes were amplified using the primers listed in Table 1. 16S rRNA was used as reference gene. Each 20 μL reaction contained 10 μL of maxima 2× SYBR green qPCR master mix (Fermentas, South Africa), 1 μL of each primer (1.5 μM), 1 μL cDNA (100 ng) and 7 μL DNase free water with the amplification conditions: 1 cycle at 95°C, 15 min; 45 cycles at 95°C, 15 s, optimum annealing temperature at 51°C for *tfdC* and 60°C for *C230* for 15 s; 1 cycle at 72°C for 30 s [7, 26].

RESULTS

Bacterial growth kinetics and degradation of 2,4-D; 2,4-DCP and 2,4,6-TCP. The peak absorbance values (OD_{600}) obtained for the isolates during growth in the presence of 2,4-D; 2,4-DCP and 2,4,6-TCP are shown in Table 2. The results show that all the strains can grow effectively in the presence of 2,4-D; 2,4-DCP and 2,4,6-TCP with the peak OD values ranging from 0.240–0.282, 0.197–0.264 and 0.228–0.300, respectively (Table 2). *P. putida* DLL-E4 and *P. fluorescens* grow most in the presence of 2,4-D (OD_{600} of 0.282 and 0.244, respectively), while *P. reactans* had the peak growth value (OD_{600} of 0.3) in the presence of 2,4,6-TCP. After 144 h of incubation at an initial 40 ppm substrate concentration, 88–94% 2,4-D (Fig. 1a), 96.9–98.2% 2,4-DCP (Fig. 1b) and 76.7–98.4% 2,4,6-TCP (Fig. 1c) were observed to be degraded by the bacterial isolates (Table 3). As shown in Table 3, *P. putida* DLL-E4 degraded 2,4-D the most with the degradation rate of 0.21 mg L^{-1} day^{-1}, while *P. fluorescens* and *P. reactans* degraded 2,4-D almost at the same rate (0.19 and 0.18 mg L^{-1} day^{-1}, respectively). *P. putida* DLL-E4 degraded 2,4-DCP

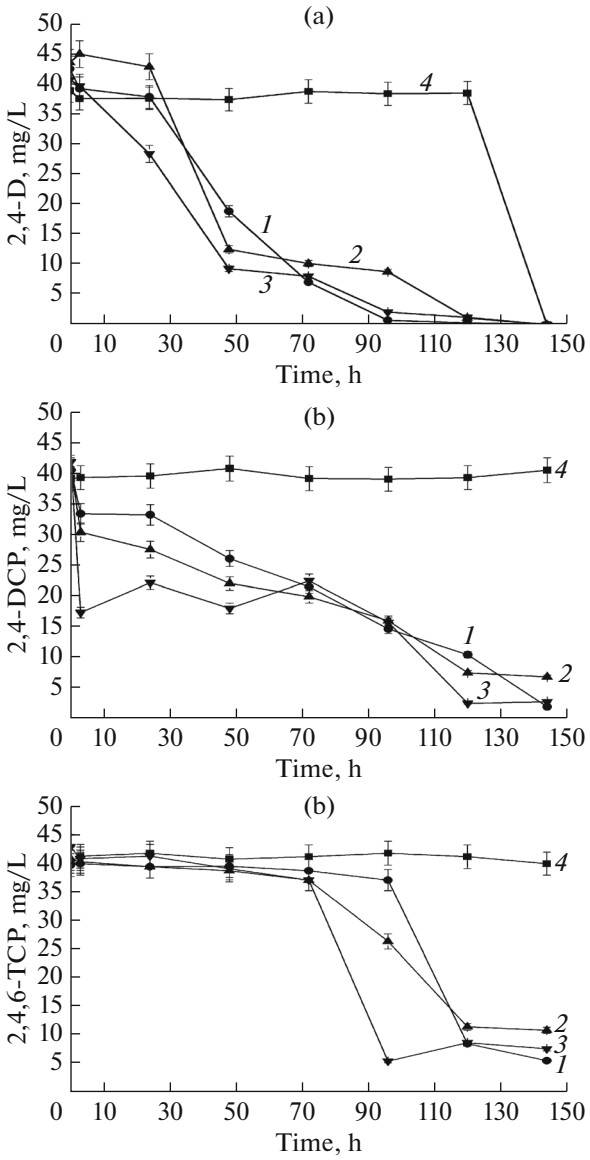

Fig. 1. Biodegradation of 2,4-D (a), 2,4-DCP (b) and 2,4,6-TCP (c) by *P. putida* DLL-E4 (*1*), *P. reactans*, (*2*), *P. fluorescens* (*3*) and negative control (*4*).

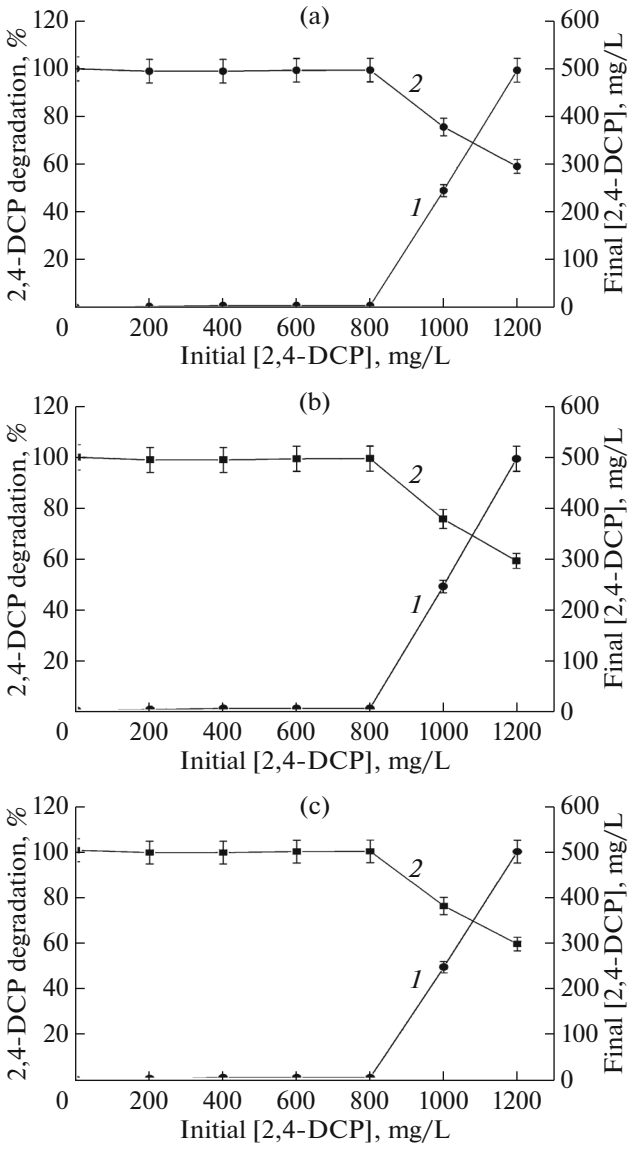

Fig. 2. 2,4-DCP concentration (*1*) and percentage 2,4-DCP (*2*) degradation after batch operation for 144 hours by *P. putida* DLL-E4 (a), *P. reactans* (b) and *P. fluorescens* (c).

faster (0.46 mg L^{-1} h^{-1}) as compared to *P. fluorescens* (0.32 mg L^{-1} h^{-1}) and *P. reactans* (0.35 mg L^{-1} h^{-1}). Similarly, 2,4,6-TCP was degraded the most by *P. putida* DLL-E4 (0.28 mg L^{-1} h^{-1}), followed by *P. reactans* (0.21 mg L^{-1} h^{-1}) and *P. fluorescens* (0.20 mg L^{-1} h^{-1}).

2,4-DCP degradation inhibition kinetics. The batch culture experiments showed that *P. putida* DLL-E4 and *P. reactans* were able to almost completely degrade 2,4-DCP up to a concentration of 800 mg/L after 144 h of incubation, with percentage degradation generally above 98% observed when the initial concentration was between 200 and 800 mg/L (Figs. 2a, 2b, respectively). *P. fluorescens* degraded 100% of 2,4-DCP up to an initial concentration of 800 mg/L (Fig. 2c). A decrease in degradation of 2,4-DCP was observed as the initial 2,4-DCP concentration increases to 1000 mg/L. The results also revealed an increase in the degradation rate when the initial 2,4-DCP concentration increased to 800 mg/L, above which a slight decrease in degradation rate of 2,4-DCP was observed due to inhibition by the substrate (Fig. 3).

Catechol dioxygenase activities and RT-PCR profile. The results for the enzyme activity assays and RT-PCR are shown in Table 4. Incubation of the cell lysates of *P. putida* DLL-E4 and *P. reactans* with the substrate catechol resulted in a much higher production of 2-hydroxymuconic semialdehyde compared to *cis,cis*-muconic acid in the reaction mixture, indicat-

Table 3. Percentage degradation and degradation rate constants at 40 ppm initial concentration of the compounds

Compound	Degradation, %, rate mg/L/h		
	P. putida DLL-E4	*P. reactans*	*P. fluorescens*
2,4-D	94.0 0.21	88.0 0.18	88.6 0.19
2,4-DCP	98.2 0.46	97.5 0.35	96.9 0.32
2,4,6-TCP	98.4 0.28	81.5 0.21	76.7 0.20

Table 4. Enzyme activities of cell extracts and maximum gene expression during the degradation of catechol

Bacterium	Maximum gene expression, -fold		Enzyme activity, μmoles/min	
	tfdC	*C230*	catechol 1,2-dioxygenase	catechol 2,3-dioxygenase
P. putida DLL-E4	0.61	3.94	0.009	0.193
P. reactans	0.74	32.66	0.029	1.090
P. fluorescence	0.36	2.66	nd*	nd*

* nd – not determined.

ing a very low catechol 1,2-dioxygenase activity. The results clearly indicate the production of catechol 2,3-dioxygenase, which is indicative that 2,4-DCP was degraded via meta-cleavage pathway by these organisms. Catechol 2,3-dioxygenase activity was 21.9-fold and 37.6-fold higher than catechol 1,2-dioxygenase activity for *P. putida* DLL-E4 and *P. reactans,* respectively. Relative quantification (RQ) of gene expression revealed between 6.5-44.2-fold increases in *C230* expression in the three organisms relative to *tfd*C, with the highest (32.7-fold) *C230* gene expression observed in *P. reactans* (Table 4). The results from the RT-PCR experiments correlated with the results from enzyme activity studies, since higher expression of *C230* corresponds to high catechol 2,3-dioxygenase activity in all the isolates, suggesting that 2,4-DCP degradation by these isolates occurred via the meta-cleavage pathway.

Relationship of the obtained isolates with *Pseudomonas* spp. degrading 2,4-DCP. According to the phylogenetic tree based on the 16S rRNA gene sequences (Fig. 4), all three *Pseudomonas* spp. from this study had a close relationship to different *P. putida* and *P. reactans* clusters. The tree shows that the 16S rRNA gene sequences of the three organisms in this study are very similar to that of *Pseudomonas* sp. 3CP (Accession Number AB908748) which was enriched from river sediment polluted with volatile organic compounds and implicated to anaerobic dechlorinate 3-chlorophenol (unpublished data). However, the isolates tested in this study were distantly related to *Pseudomonas* sp. 1087 (Accession Number AY230196), *Pseudomonas* sp. 1131 (Accession Number AY230195) and *P. aeruginosa* GF (Accession Number KM259920), all implicated in the degradation of chlorophenolic compounds (unpublished data).

DISCUSSION

The metabolic ability demonstrated by indigenous bacteria for degradation of toxic compounds in polluted environment may indicate the development of the various catabolic pathways that allows these organisms to adapt to the toxic environment [27]. The bioremediation strategy in removing these toxic compounds based on their use as suitable carbon and energy sources, by microorganisms is one of the main focuses of environmental pollution control [28]. This strategy was tested in this study by using the chlorophenolic compounds as the sole carbon and energy source for use by the indigenous *Pseudomonas* sp. iso-

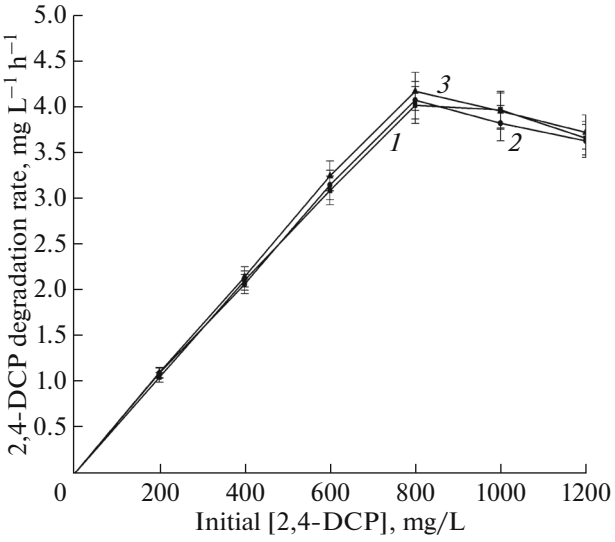

Fig. 3. Relationship between initial 2,4-DCP concentration and rate of 2,4-DCP degradation by *P. putida* DLL-E4 (*1*), *P. reactans* (*2*) and *P. fluorescens* (*3*).

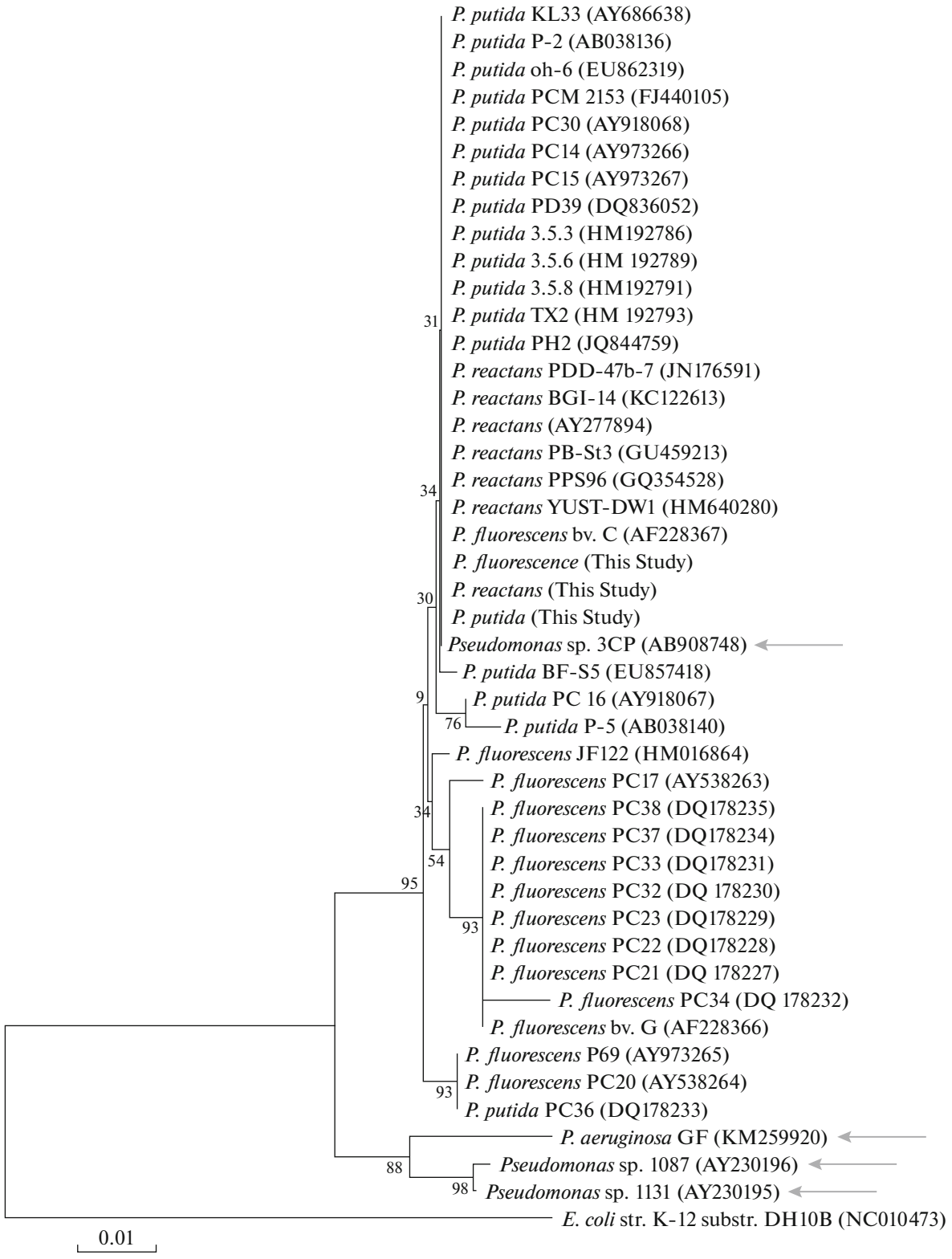

Fig. 4. Phylogenetic tree showing evolutionary relationships of *P. putida* DLL-E4, *P. reactans* and *P. fluorescens* (in this study) to 44 *Pseudomonas* spp. reported in literature to degrade chlorophenol and other phenolic compounds based on their 16S rRNA gene sequences. The tree was generated with the neighbor-joining method with 1000 bootstrap re-samplings. Strains indicated with a red arrow have been previously described to degrade chlorophenolic compounds.

lates. Various factors have been implicated to affect the ability of indigenous bacterial isolates to degrade toxic compounds, including high toxicity levels and low degradability potential of the compounds as well as the growth inhibition of cells during the degradation process [29]. In the present study, the ability of some indigenous bacterial isolates obtained from chlorophenol-contaminated soil samples collected from a sawmill located in Durban (South Africa) effectively to degrade 2,4-D; 2,4-DCP and 2,4,6-TCP in batch cultures was investigated, and the possible degradation pathway for 2,4-DCP was proposed based on the results of the catechol dioxygenase activity assay and gene expression study.

The phenol hydroxylase gene is involved in the first step in the catabolism of phenol which involves the hydroxylation of phenol to catechol by the addition of a single hydroxyl group to the substrate. This step is then followed by ring cleavage, which produces intermediates that can then be easily converted to metabolites of the Krebs cycle [30]. *P. pseudoalcaligenes* strain MH1 has been reported to express phenol hydroxylase and degrade phenol at a concentration of 1.5 g/L [25]. The *Pseudomonas* spp. isolated and tested in this study showed promising results for 2,4-D; 2,4-DCP and 2,4,6-TCP degradation while utilizing the compounds as the sole carbon and energy source for growth.

Previous studies have shown that *Pseudomonas* spp. possess the ability to degrade and grow on many aromatic compounds such as 2,4,6-TCP; pentachlorophenol, nitrified aromatic compounds and polycyclic aromatic hydrocarbons [31]. This is corroborated by the results obtained in this study demonstrating the ability of the *Pseudomonas* spp. to use 2,4-D; 2,4-DCP and 2,4,6-TCP as sole carbon source. *P. putida* DLL-E4 demonstrated the highest peak growth values in the presence of 2,4-D and 2,4-DCP compared to the other two test isolates (Table 2). Also, *P. putida* DLL-E4 was able to degrade 98.2, 98.4 and 94.0% of 2,4-D, 2,4-DCP and 2,4,6-TCP, respectively. Previous studies involving *P. fluorescens* [25] showed effective degradation of phenol; it is therefore not surprising that the *P. fluorescens* isolated in the current study demonstrated higher capability for the degradation of phenol than the previous report [28].

Biodegradation of chlorinated aromatic compounds usually decreases with an increase in the number of chlorine groups [32]. This trend can be seen from the results of the current study, where all the bacterial isolates degraded a lower percentage of 2,4,6-TCP in comparison to 2,4-D and 2,4-DCP, except for *P. putida* DLL-E4 showing similar percentage degradation for both 2,4-DCP and 2,4,6-TCP. Overall, *Pseudomonas* spp. proved to be excellent degraders of 2,4-D; 2,4-DCP and 2,4,6-TCP showing a minimum degradation of 76.4% and a maximum of 98.4% during the specified experimental duration. Increased concentrations of chlorophenolic compounds are known to be inhibitory to the growth and degradation ability of microorganisms [8]. Addition of glucose to the media has been shown to improve the metabolism of the chlorophenols by means of NADH production required by the monooxygenase enzyme involved in the breakdown process [24, 33]. In the present study, glucose was added as a supplementary component to allow *P. putida* DLL-E4*, P. reactans* and *P. fluorescens* to effectively degrade very high concentrations of 2,4-DCP over a period of 6 days. The degradation assays showed that *P. putida* DLL-E4 and *P. reactans* were able to almost completely degrade 2,4-DCP even at the initial concentration of 600 ppm (Figs. 2a, 2b). However, concentrations greater than 600 ppm were shown to reduce the degradation efficiency of these species. Similarly, *P. fluorescens* was able to almost completely degrade 2,4-DCP up to a concentration of 800 ppm (Fig. 2c). Results obtained for the substrate inhibition kinetics were similar to those previously reported [34]. It can also be noted that in shake-flask cultures, the percentage 2,4-DCP degradation decreased with increasing initial concentration of compound. The degradation assay showed that at lower 2,4-DCP concentrations, the microorganisms were able to degrade greater amount of the substrate than at higher concentrations. This may be due to the toxicity of 2,4-DCP to these bacterial isolates at higher concentration, resulting in decrease in growth and degradation abilities.

In *Pseudomonas* spp., many induced enzymes are nonspecific and the metabolic pathway contains a high level of convergences which allows for efficient utilization of many growth substrates without repeating genetic coding for enzymes [25]. Aerobic degradation of chlorophenolic compounds involves the ring hydroxylation of neighboring carbon atoms as well as the ring cleavage of the resulting catecholic intermediates. With phenol degradation, the ring structure is monohydroxylated by phenol hydroxylase at the ortho-position. Thereafter, the reaction is catalyzed by either catechol 1,2-dioxygenase (involving the ortho-pathway), forming succinyl-CoA and acetyl-CoA or catechol 2,3-dioxygenase (involving the meta-pathway), forming pyruvate and acetaldehyde [35]. Chlorophenols (particularly 2,4-D and 2,4-DCP) are degraded via the meta- or ortho- pathways. In this study, real-time PCR was used to observe the presence and relative expression of the two genes (*tfd*C and *C230*) involved in 2,4-DCP degradation. The *tfdC* gene encodes for a chlorocatechol 1,2-dioxygense enzyme, while the *C23O* gene encodes for catechol 2,3-dioxygenase [7]. The primers used in this study are specifically designed to detect the bacterial populations capable of degrading aromatic compounds via catechol cleavage pathways [7, 26]. Results obtained from real-time PCR (Table 4) revealed high expression of gene (*C23O*) encoding meta-cleaving enzymes for all the *Pseudomonas sp* isolates tested in this study. This was further confirmed by the enzyme assays which showed the induction of cate-

chol 2,3-dioxygenase enzyme in the cell lysates of the isolates (Table 4). The results indicated an elevated enzyme activity for the production of 2-hydroxymuconic semialdehyde, indicative of catechol 2,3-dioxygenase expression. The observed 21.91- and 37.59-fold higher expression of catechol 2,3-dioxygenase activity than that of catechol 1,2-dioxygenase for *P. putida* DLL-E4 and *P. reactans,* respectively (Table 3) is similar to the findings of Mahiudddin with coauthors [25], where 21-fold higher activity of catechol 2,3-dioxygenase was reported for *Pseudomonas* sp.

The present study shows that the indigenous *Pseudomonas* spp. isolates obtained from chlorophenol-contaminated soil samples collected from a sawmill located in Durban (South Africa) had the ability to efficiently utilize 2,4-D; 2,4-DCP and 2,4,6-TCP as sole carbon sources. At a low concentration (40 mg/L), the isolates were capable of degrading about 95% of the compounds over a period of 6 days. Amongst the three isolates, *P. putida* DLL-E4 was the best degrader of 2,4-D; 2,4-DCP and 2,4,6-TCP with the highest peak growth values, percentage degradation and degradation rate. In addition, the isolates could tolerate and degrade very high concentrations of 2,4-DCP in the presence of glucose. All three isolates follow the meta-cleavage degradation pathway based on the results obtained from the RT-PCR and enzyme activity assays. Further optimization of the organisms for enhanced degradation of chlorophenolic compounds and proper elucidation of the degradation pathways followed by these organisms for the respective compounds is currently ongoing in our laboratory.

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