Isolation and characterisation of endocrine disruptor nonylphenol-using bacteria from South Africa

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Endocrine disrupting chemicals (EDCs) are synthetic chemicals that alter the function of endocrine systems in animals including humans. EDCs are considered priority pollutants and worldwide research is ongoing to develop bioremediation strategies to remove EDCs from the environment. An understanding of indigenous microorganisms is important to design efficient bioremediation strategies. However, much of the information available on EDCs has been generated from developed regions. Recent studies have revealed the presence of different EDCs in South African natural resources, but, to date, studies analysing the capabilities of microorganisms to utilise/degrade EDCs have not been reported from South Africa. Here, we report for the first time on the isolation and enrichment of six bacterial strains from six different soil samples collected from the Mpumalanga Province, which are capable of utilising EDC nonylphenol as a carbon source. Furthermore, we performed a preliminary characterisation of isolates concerning their phylogenetic identification and capabilities to degrade nonylphenol. Phylogenetic analysis using 16S rRNA gene sequencing revealed that four isolates belonged to *Pseudomonas* and the remaining two belonged to *Enterobacteria* and *Stenotrophomonas*. All six bacterial species showed degradation of nonylphenol in broth cultures, as HPLC analysis revealed 41–46% degradation of nonylphenol 12 h after addition. The results of this study represent the beginning of identification of microorganisms capable of degrading nonylphenol, and pave the way for further exploration of EDC-degrading microorganisms from South Africa.

Significance:
- First report of endocrine disruptor nonylphenol-using bacteria from South Africa
- Six bacterial species capable of using nonylphenol as a carbon source were isolated
- Results will pave the way for further exploration of endocrine disruptors degrading microbes from South Africa

Introduction

Endocrine disruptors or endocrine disrupting chemicals (EDCs) are chemicals that can alter the functioning of endocrine systems in humans and other animals including wildlife, and can thus cause cancerous tumour development, birth defects and other developmental disorders.[1,2] Many chemicals have been identified as EDCs, and many are used in the formulation of various pharmaceutical products, pesticides, industrial chemicals, heavy metals, persistent organochlorines and other organohalogens, alkylphenols, and synthetic and natural hormones.[2,3] These environmental pollutants mimic natural hormones of the endocrine system and display either oestrogenic or androgenic activities.[1,2,4] They can thus have adverse effects by either unnaturally inhibiting or stimulating the endocrine system and/or hormonal production.[1,2,4] Exposure to EDCs increases the chance of physiological abnormalities and alters cognitive function in animals, including humans.[1,2] Physiological abnormalities include low sperm count and decreased sperm quality[5], as well as premature puberty in both girls[6] and boys[7]. Several other metabolic disorders have been reported, including different types of cancers and thyroid-related problems including obesity.[1,2,8]

Investigations have also shown that these types of chemicals also affect other animals. Effects of EDCs on aquatic species have been well documented.[9] EDCs have been reported to have adverse effects on invertebrates and wildlife populations.[10] Female snails exposed to tributylin exhibited masculinisation (a disorder called imposex in which female snails develop a male sex organ, including a penis and vas deferens), which in turn led to a decline in the population.[11] Alligators of Lake Apopka (Florida, USA) were reported to have impaired sexual development and function as a result of exposure to dichlorodiphenyltrichloroethane (DDT).[12] Exposure to dichlorodiphenyldichloroethylene (DDE) resulted in a decline in numbers of bald eagles in Europe and North America.[13]

To date, information concerning EDCs has been primarily derived from studies conducted in developed countries.[2] Much information is still, however, lacking from large parts of Africa, Asia and Central and South America.[2] Studies on EDCs from South Africa in particular are very scarce. A report presented by the Water Research Commission of South Africa revealed the presence of EDCs in South African water.[14] In addition to this report, studies conducted in a few places within South Africa have also revealed the presence of EDCs. DDT, DDE and phthalate esters have been found in Limpopo[15-17]; oestrone, oestradiol and oestriol (steroids hormones) in the Western Cape[18] and in KwaZulu-Natal[19]; *p*-nonylphenol, diethylhexyl phthalate and dibutyl phthalate in Gauteng[20]; and lastly DDT, chlordane, hexachlorobenzene, heptachlor and endosulfan in the Eastern Cape[21]. In addition, a large number of EDCs was found in upstream and downstream sections of wastewater treatment plants.[22,23]

As a result of their adverse effects on humans and wildlife, EDCs are considered to be priority pollutants, and worldwide research is ongoing to develop remediation strategies to remove these chemicals from the environment.

Strategies for removal – including advanced oxidation processes[24], electrochemical separation and degradation technologies[25] and bio-remediation and combinatorial techniques[26,27] – have been extensively investigated. Bioremediation is a particularly attractive approach, as it represents natural and economically feasible processes for detoxification of environmental pollutants under environmental conditions. An understanding of indigenous microorganisms is therefore important to facilitate the design of efficient bioremediation strategies. However, to date, studies on the analysis of the capabilities of microorganisms to utilise/degrade EDCs have not been reported from South Africa. This study is the first of its kind on the enrichment, isolation, identification and further assessment of the EDC-degradation capability of bacteria from South African soils.

Materials and methods

Soil sample collection and preparation

Soil samples were aseptically collected from soil at different coal-fired power stations in and around the Mpumalanga Province, South Africa. The selected sampling areas are represented in a schematic diagram with GPS coordinates (Figure 1). Soil samples (5 g) were re-suspended in 30 mL of DNase-free and RNase-free water.[28,29] The samples were vigorously vortexed for 5 min, followed by incubation on a rotary shaker for 1 h at room temperature at 100 rpm.[28,29] After incubation, the soil was allowed to settle out of solution (30 min), and the supernatants were collected and immediately used for isolation of microorganisms.

Medium preparation

All chemicals and reagents used in this study were purchased from Sigma-Aldrich (Johannesburg, South Africa), unless otherwise stated. Minimal medium[28,29] with added trace element solution[30] was used for isolation of microorganisms. The minimal medium consisted of 8.5 g/L $Na_2HPO_4.2H_2O$, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 0.5 g/L $MgSO_4.7H_2O$, 14.2 mg/L $CaCl_2$ and 0.15 g/L KCL. The minimal medium was supplemented with 10 mL of trace element solution[30], consisting of 0.4 mg/L $CuSO_4$, 1.0 mg/L KI, 4.0 mg/L $MnSO_4.H_2O$, 4.0 mg/L $ZnSO_4.7H_2O$, 5.0 mg/L H_3BO_3, 1.2 mg/L $Na_2MO_4.2H_2O$ and 2.0 mg/L $FeCl_3.6H_2O$, per litre of medium. Technical grade nonylphenol (catalogue number 290858) was added as a sole source of carbon to a final concentration of 5 mM.

Enrichment procedure

Supernatant (1 mL) from the soil samples was used to inoculate 100 mL of minimal medium in a 500-mL conical flask, supplemented with nonylphenol as the sole carbon source. A control was set up to contain medium and nonylphenol, without inoculation of soil samples. After 4 weeks of incubation at 37 °C at 100 rpm, 1 mL of culture was used to inoculate fresh minimal medium (100 mL) with nonylphenol as the sole carbon source. This serial enrichment of bacterial isolates was repeated until a single, homogenous culture was obtained. Aliquots (100 μL) of cultures were spread on minimal medium agar plates with nonylphenol (5 mM) as the sole carbon source, to monitor the growth of microorganisms at 37 °C. The minimal medium plates with nonylphenol were prepared as described elsewhere.[31] Bacterial growth was also analysed by measuring the absorbance at 600 nm.

Isolation of genomic DNA and amplification of 16S rRNA gene

Genomic DNA (gDNA) from bacterial isolates was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit (catalogue number D6005, Inqaba Biotec, Pretoria, South Africa) according to the manufacturer's protocol.

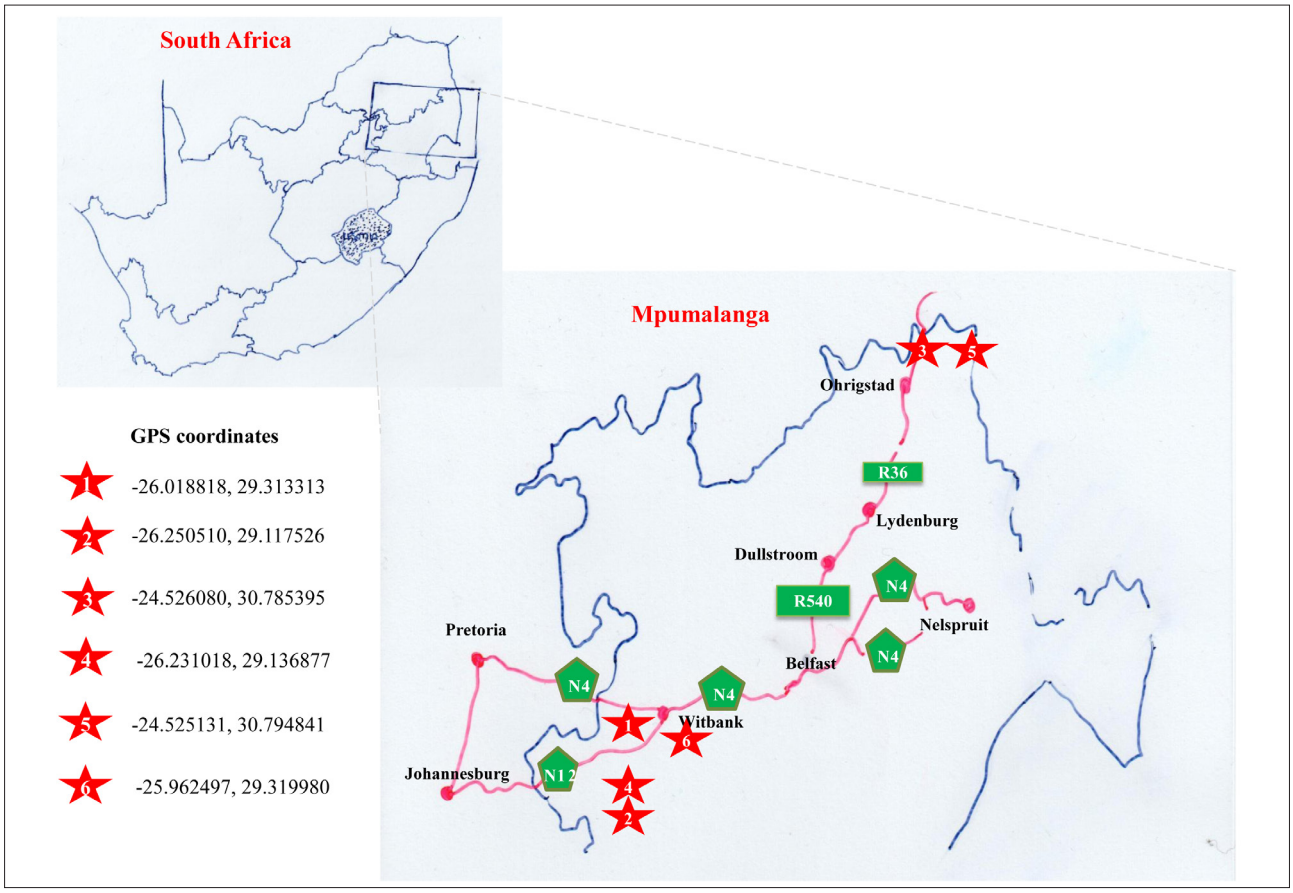

Figure 1: Schematic representation of soil sample collection areas in Mpumalanga, South Africa. The numbers 1 to 6 in stars indicate the areas from which the soil samples were collected. The GPS coordinates of the sampling areas are given in the figure.

The gDNA was visualised using agarose gel electrophoresis, and gDNA concentration was measured using a SimpliNano microvolume spectrophotometer (catalogue number GE29-0617-12, Sigma-Aldrich, St. Louis, MO, USA). The isolated gDNA was used for amplification of the 16S rRNA gene. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using primers 63f and 1387r as described elsewhere.[32] A KAPA HiFi HotStart PCR kit (catalogue number KK2501, KAPA Biosystems, Wilmington, MA, USA) was used to amplify the 16S rRNA gene according to manufacturer's instructions. The PCR products were run on a 0.8% agarose gel and were purified using the Wizard® SV Gel and PCR Clean-Up System (catalogue number A9281, Promega, Madison, WI, USA).

16S rRNA gene sequencing

Samples were prepared for sequencing using the BigDye™ Terminator V3.1 Cycle Sequencing Kit (catalogue number 4337455, Thermo Fischer Scientific, Waltham, MA, USA). The aforementioned primers 63f and 1387r[32] were used for sequencing. The sequencing reactions were performed according to the parameters described by the manufacturer. Sequencing reactions were purified using the EDTA-ethanol method described by the manufacturer, and submitted for sequencing using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Consensus sequences were derived from the sequences obtained from the forward and reverse primer reactions for each product, using Geneious® R9 9.1.2. software.

Phylogenetic analysis

16S rRNA gene sequences of bacterial isolates were subjected to BLAST analysis at NCBI (the US National Center for Biotechnology Information) against 16S ribosomal RNA sequences (Bacteria and Archaea) to identify the closest homologs. Among the resulting hits, the 16S rRNA sequences with 100% or 99% identity homologs were selected. Based on the obtained bacterial species, the type strains belonging to each species were selected, and the 16S rRNA sequences were retrieved from elsewhere (http://www.bacterio.net/). The *Escherichia coli* ATCC 11775 type strain 16S rRNA gene sequence (also retrieved from http://www.bacterio.net/) was used as an out-group. Phylogenetic analysis was carried out using the maximum likelihood method based on the Tamura–Nei model.[33] Initial tree(s) for the heuristic search were obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.[34] Phylogenetic analysis included the isolate 16S rRNA gene sequence, hit homologs and type strain 16S rRNA gene sequences. The phylogenetic tree was presented with branch lengths, and the bacterial isolates identified in this study are highlighted in bold font.

Nonylphenol degradation

A degradation study using whole cells was carried out as described elsewhere[35,36] to assess the capabilities of the bacterial isolates to degrade nonylphenol. A single colony of isolates from minimal medium plate containing nonylphenol as the carbon source was used to inoculate 5 mL of Luria–Bertani broth, which was then cultured overnight at 150 rpm at 37 °C. The growth of the isolates was measured at 600 nm after diluting the culture in Luria–Bertani broth. The cultures were then washed twice with saline (0.9% sodium chloride solution), followed by inoculation with an equal amount of each overnight bacterial culture for all six isolates onto separate, fresh minimal media (5 mL) containing nonylphenol (2.5 mM) as a carbon source in 50-mL glass tubes (test cultures). The test cultures were incubated for 12 h at 37 °C at 150 rpm. After incubation, 5 mL of ethyl acetate was added to the test cultures, which were then vortexed for 5 min at maximum speed, followed by centrifugation for 5 min at 2500 *g* at room temperature. After centrifugation, two distinct fractions were separated by a thin middle layer composed of bacterial cell debris. The upper organic fraction containing nonylphenol was removed from the lower aqueous fraction into a fresh glass tube. The extraction was repeated twice, followed by evaporation of the organic fraction. The remaining residue was re-suspended in 200 μL of HPLC-grade methanol. Minimal medium with nonylphenol but without culture was used as a control and treated the same as the test culture.

HPLC analysis of nonylphenol was carried out following the method described elsewhere, with modifications.[35,36] Briefly, the abovementioned methanol samples were filtered through 0.45-μm glass fibre filters and analysed using a Shimadzu Prominence instrument (Shimadzu, Roodepoort, South Africa) equipped with a C18 analytical column (4.6 mm × 250 mm; particle size 5 μm from Sigma-Aldrich, South Africa) and with a dual wavelength UV/Vis detector. Separation was achieved using a 22.5-min linear gradient of acetonitrile in water (50% to 96.5%, and then re-equilibrated for 10 min at 50% acetonitrile at a flow rate of 1.25 mL/min). A volume of 5 μL of sample was injected for analysis. Nonylphenol was detected at 277 nm, and the percentage degradation of nonylphenol by test cultures was related to the control nonylphenol, which was taken as 100%.

Statistical analysis

All experiments were carried out in triplicate and results were subjected to statistical analysis as described elsewhere.[35,36] The activities, in terms of percentage degradation, of the different bacterial isolates were analysed for means and standard deviations and compared for statistical differences using a Student's *t*-test on GraphPad QuickCalcs software package (GraphPad Software Inc., CA, USA).

16S rRNA gene sequences accession numbers

16S rRNA gene sequences of bacterial isolates identified in this study were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/), with the following accession numbers: KX364074 (*Pseudomonas nitroreducens* strain LBQSKN1), KX364075 (*Pseudomonas putida* strain LBQSKN2), KX364076 (*Stenotrophomonas* sp. LBQSKN3), KX364077 (*Enterobacter asburiae* strain LBQSKN4), KX364078 (*Pseudomonas* sp. LBQSKN5) and KX364079 (*Pseudomonas* sp. LBQSKN6).

Results and discussion

Enrichment and isolation of nonylphenol-utilising bacteria

The sampling areas selected for this study (represented in Figure 1) have been reported to harbour polycyclic aromatic hydrocarbons (PAHs).[37] PAHs are hydrophobic compounds well known for their carcinogenicity and mutagenicity towards humans.[38,39] In this study, we aimed to test the ability of bacterial species growing in the presence of PAHs to degrade EDCs, as these chemicals are also hydrophobic and aromatic in nature. To isolate microorganisms capable of utilising nonylphenol as a sole source of carbon, we followed a standard enrichment method. Soil samples collected from six different places (Figure 1) were inoculated into minimal medium supplemented with nonylphenol as a carbon source. After 4 weeks of incubation, growth of bacteria was observed on minimal medium plates supplemented with nonylphenol as a carbon source, as well as assessed through spectrophotometry. The initial bacterial growth on plates was non-homogenous, suggesting the presence of more than one type of species. After three successive serial cultures, a homogenous population of bacteria was observed on minimal medium plates, indicating that successive serial culturing resulted in the enrichment of a single type of bacteria that are capable of utilising nonylphenol as a sole source of carbon. In this study, six bacteria were isolated from the six different soil samples.

Identification of bacterial isolates

In order to identify the enriched bacterial isolates, 16S rRNA gene sequence-based phylogenetic analysis was carried out. The 16S rRNA genes from the gDNA of bacterial isolates were PCR amplified using the 63f and 1387r primer set as described elsewhere.[32] Analysis of the PCR amplified products on agarose gel showed prominent DNA bands with approximate sizes of ≥1200 base pairs (Figure 2). This analysis indicates specific amplification of the 16S rRNA gene. The amplified 16S rRNA gene was gel purified and subjected to sequence analysis using the same primers used for its amplification. Sequence analysis was performed using both forward and reverse primers, yielding a consensus sequence of 300–500 overlapping base pairs between the sequences. The sizes of the 16S rRNA sequences obtained for each of the bacterial isolates are presented in Table 1. The 16S rRNA sequence of Isolates 1 and 2 showed 100% identity to *Pseudomonas* spp., while Isolates 5

and 6 also had 99% identity to *Pseudomonas* spp. (Table 1). Isolate 3 showed 99% identity to *Stenotrophomonas* spp. and Isolate 4 showed 99% identity to *Enterobacter* spp. This indicates that most of the isolates belong to *Pseudomonas* (Table 1). Phylogenetic analysis of isolates based on 16S rRNA gene sequences compared to the 16S rRNA gene sequences of hit species, highlighted the differential alignment of bacterial isolates with different species (Figure 3). Based on the phylogenetic alignment, the six bacterial isolates were named as shown in Table 1. Furthermore, homology analysis (per cent identity) of 16S rRNA gene sequences among bacterial isolates (Table 2) revealed that Isolates 3 and 4 have low per cent identity compared with that of the other isolates, clearly reinforcing that they in fact belong to different bacterial genera. Species assigned to *Pseudomonas* on the other hand showed high per cent identity (Table 2), demonstrating that they belong to the same genus.

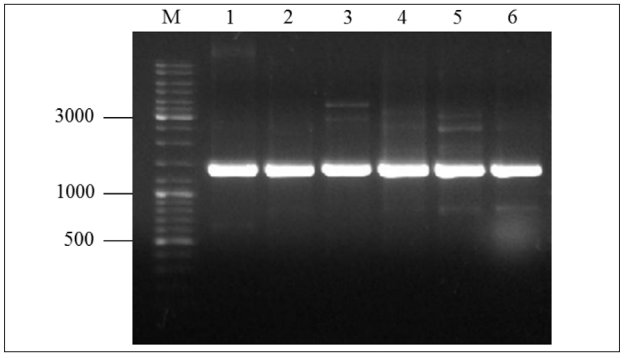

Figure 2: Agarose gel electrophoresis analysis of 16S rRNA genes amplified from six bacterial isolates. PCR amplified products were run on 1% agarose gel. Lane M indicates the DNA ladder (O'GeneRuler DNA Ladder Mix 100–10 000 base pair, catalogue number SM1173, ThermoFisher). Markers with high intensity were indicated by their size. Lanes 1 to 6 indicate the PCR amplified 16S rRNA gene of the respective bacterial isolates.

Degradation of nonylphenol by bacterial species

Whole-cell nonylphenol degradation experiments were carried out to assess the nonylphenol degradation capability of each bacterial isolate. As shown in Figure 4, all bacterial isolates showed degradation of nonylphenol. The degradation of nonylphenol by bacterial isolates ranged from 41% to 46% (Figure 4). However, the difference in percentage of nonylphenol degradation by all six bacterial species was considered to be the same, because the percentage differences among the isolates was not statistically significant ($0.2<p<0.7$). Nonylphenol degradation by the bacterial species identified in this study is reinforced by the literature. Species belonging to the genus *Pseudomonas* have been shown to degrade EDCs such as di-*n*-butyl phthalate[40], *p*-nonylphenol[41] and polyethoxylated nonylphenols[42,43]. Bacterial species belonging to *Stenotrophomonas* were previously found to be capable of using either nonylphenol or octylphenol as a sole carbon source.[44] For species belonging to the well-known human-pathogenic and plant association *Enterobacter*, degradation of EDCs has been reported particularly for bisphenol A[45], polychlorinated biphenyls[46], endosulfan[47], dibutyl phthalate[48] and nonylphenol[49].

All of the bacterial species isolated in this study also have the capability to degrade PAHs. PAH degradation by *Pseudomonas* species is well reported.[50-54] Degradation of PAHs using *Stenotrophomonas*[55], in particular *Stenotrophomonas maltophilia*[56-58], has been investigated. Hydrocarbon degradation capabilities for some of these species have also been demonstrated with aliphatic[59] and aromatic hydrocarbons[60]. This suggests that the soil samples used in this study, from areas where PAHs were reported to be present, harbour bacterial species that are capable of degrading both classes of xenobiotics, PAHs and EDCs.

Conclusion

The distribution of EDCs, their effects towards living organisms and microorganisms capable of degrading ECDs, and the mechanisms of EDC degradation have been thoroughly documented by the developed world. Information on these matters is, however, lacking from Africa, Asia and Central and South America.

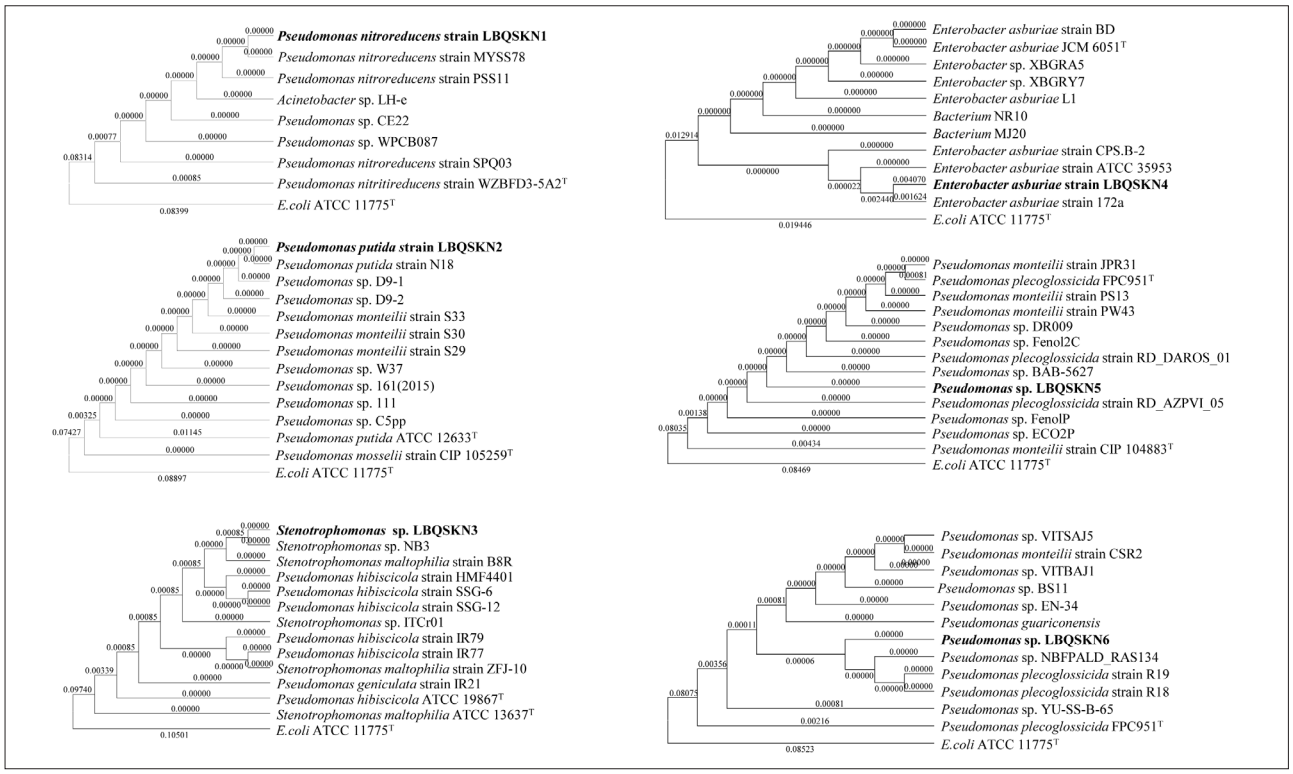

Figure 3: Phylogenetic analysis of the 16S rRNA gene sequences of the bacterial isolates. 16S rRNA gene sequences of the type strains belonging to the same genus and an out-group bacterial species (*E. coli*) were also included in the analysis. Superscript letter 'T' next to strain name indicates the type strain. Each bacterial isolate was named based on its alignment to the homolog bacterial species. Branch lengths are also shown in the tree. Bacterial species isolated and named in this study are highlighted in bold font.

Table 1: Bacterial isolates identified

Sample	16S rRNA sequenced gene size (base pair)	GenBank accession number	NCBI Blast hit results			Name assigned to the bacterial isolate
			Dominant bacteria genus	% Identity	% Query cover	
1	1242	KX364074	*Pseudomonas*	100	100	*Pseudomonas nitroreducens* strain LBQSKN1
2	1239	KX364075	*Pseudomonas*	100	100	*Pseudomonas putida* strain LBQSKN2
3	1196	KX364076	*Stenotrophomonas*	99	100	*Stenotrophomonas* sp. LBQSKN3
4	1240	KX364077	*Enterobacter*	99	100	*Enterobacter asburiae* strain LBQSKN4
5	1245	KX364078	*Pseudomonas*	99	100	*Pseudomonas* sp. LBQSKN5
6	1237	KX364079	*Pseudomonas*	99	100	*Pseudomonas* sp. LBQSKN6

NCBI, US National Center for Biotechnology Information

Table 2: Homology (percentage identity) analysis of 16S rRNA gene sequences of bacterial isolates

	***Pseudomonas nitroreducens* strain LBQSKN1**	***Pseudomonas putida* strain LBQSKN2**	***Stenotrophomonas* sp. LBQSKN3**	***Enterobacter asburiae* strain LBQSKN4**	***Pseudomonas* sp. LBQSKN5**	***Pseudomonas* sp. LBQSKN6**
Pseudomonas nitroreducens strain LBQSKN1	100.00	96.29	85.08	82.33	96.44	95.79
Pseudomonas putida strain LBQSKN2	96.29	100.00	85.41	83.63	99.84	99.51
Stenotrophomonas sp. LBQSKN3	85.08	85.41	100.00	80.69	85.41	85.26
Enterobacter asburiae strain LBQSKN4	82.33	83.63	80.69	100.00	83.83	83.63
Pseudomonas sp. LBQSKN5	96.44	99.84	85.41	83.83	100.00	99.35
Pseudomonas sp. LBQSKN6	95.79	99.51	85.26	83.63	99.35	100.00

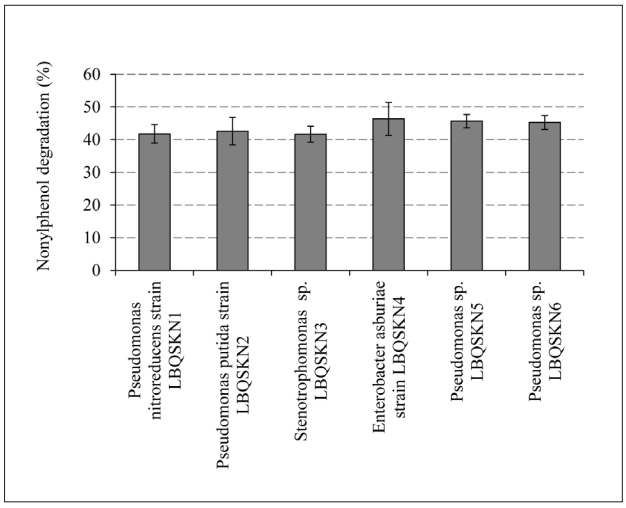

Figure 4: Analysis of nonylphenol degradation by bacterial isolates. Percentage degradation of nonylphenol by bacterial isolates was related to the control nonylphenol, which was taken as 100% as described elsewhere[35,36]. The values represent mean±s.d. for three biological replicates. Percentage degradation among different bacterial isolates was found to be not statistically significant ($0.2<p<0.7$).

Our study is thus the first of its kind from South Africa, in which we successfully enriched, isolated, identified and demonstrated nonylphenol degradation capabilities of indigenous bacterial strains. The areas from which soil samples were collected were previously reported to be polluted with PAHs, and their selection resulted in the isolation of bacterial species capable of degrading EDC nonylphenol, suggesting that these organisms have the capability to degrade a variety of xenobiotic chemicals. Further investigations on the capacity of the isolates to degrade different EDCs and PAHs are currently underway. The results presented in this study will lead to the isolation and characterisation of microorganisms from different parts of South Africa that are capable of degrading different EDCs, and will thus enrich EDC-related information from Africa.

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Authors' contributions

K.S. and C.W.T. conceived and designed the experiments. K.S. provided funding for the study. All authors were involved in performing the experiments, analysing the data and writing the manuscript.

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