Assessment of genetic diversity and bioremediation potential of pseudomonads isolated from pesticide-contaminated artichoke farm soils

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Received: 29 December 2017 / Accepted: 23 April 2018 / Published online: 19 May 2018
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Abstract

A total of 68 dimethoate and pentachlorophenol-tolerant rhizobacteria, isolated from a pesticide-contaminated agricultural soil, have been identified and typed by means of 16S–23S rRNA internal transcribed spacers analysis (ITS-PCR), 16S rRNA gene sequencing and by repetitive extragenic palindromic (BOX-PCR). The majority of bacterial isolates (84.31%) belonged to Proteobacteria (with a predominance of Gammaproteobacteria, 72.54%), while the remaining isolates were affiliated with Firmicutes (9.80%), Bacteroidetes (1.96%) and Actinobacteria (3.92%). The pesticide-tolerant bacterial isolates belonged to 11 genera, namely *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, *Comamonas*, *Achromobacter*, *Rhodococcus*, *Ochrobactrum*, *Aquamicrobium*, *Bordetella* and *Microbacterium*. Within the well-represented genus *Pseudomonas* ($n=36$), the most common species was *Pseudomonas putida* ($n=32$). The efficacy of the selected strain, *Pseudomonas putida* S148, was further investigated for biodegradation of pentachlorophenol (PCP) in minimal medium, when used as a sole carbon and energy source. At an initial concentration of 100 mg/L, *P. putida* S148 degraded 91% of PCP after 7 days. GC–MS analyses revealed the formation of tetrachlorohydroquinone, tri- and di-chlorophenols as biodechlorination products in PCP remediation experiments. The toxicity estimation showed that 50% lethal concentration (LC50) and 50% growth inhibition concentration (IGC50) obtained values for the major identified compounds (2,3,4,6 tetrachlorophenol, 2,3,5,6 tetrachlorophenol and tetrachlorohydroquinone) were higher than those estimated for the PCP indicating that the metabolites are less toxic than the original compound for those specific organisms. S148 strain could be added to pesticide-contaminated agricultural soils as a bacterial inoculant for its potential to improve soil quality.

Keywords Pesticides · Bioremediation · *Pseudomonas* · Dimethoate · Pentachlorophenol

Introduction

Pesticides are extensively used in agriculture to prevent or reduce crop losses caused by pests. Approximately 7.7 billion pounds of pesticides are applied to crops worldwide per year (Bajak 2016). Synthetic pesticides include organochlorines, organophosphates, organometallic compounds, organocarbamates, pyrethroids, among others (Gilden et al. 2010). Organochlorine and organophosphorus pesticides (OCP and OPP) are very poisonous and raise serious concerns and negative effects on food safety, human health and environmental protection. The chemical structures of these pesticides, along with their incorrect preparation, application, and storage, may pose a significant toxicity risk to many organisms (Fantke et al. 2012; Torres et al. 2013; Pieterse et al. 2015). Between 1 and 5 million

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persons per year are intoxicated with pesticides, as cited by the World Health Organization (Nabih et al. 2017). More than 500 pesticide formulations are presently applied in agricultural fields (Douglas et al. 2018). These artificially synthesized substances, that are generally nonbiodegradable, persist in agricultural fields after their application. Biodegradation mediated by indigenous microorganisms to the contaminated sites could help reduce pesticide persistence in the environments (Lal et al. 2010).

Bioremediation is considered a safe economical, efficient and sustainable technology for restoring the pesticide-contaminated sites (Rayu et al. 2012). The bacterial classes of Gammaproteobacteria (*Pseudomonas*, *Aerobacter*, *Acinetobacter*, *Moraxella*, *Plesiomonas*), Betaproteobacteria (*Burkholderia*, *Neisseria*), Alphaproteobacteria (*Sphingomonas*), Actinobacteria (*Micrococcus*), and Flavobacteria (*Flavobacterium*) are considered as highly active pesticide-degrading microorganisms (Matsumoto et al. 2008; Rao and Wani 2015). It was further reported that some bacterial strains from the genus *Pseudomonas* could degrade OPPs including chlorpyrifos, parathion, triazophos, etc. (Shi et al. 2015; Dellai et al. 2016). A number of *Pseudomonas* strains able to remediate individually or in consortium OCPs such as pentachlorophenol (PCP) have been reported, especially for *P. putida* SKG-1 MTCC (Garg et al. 2013); *P. stutzeri* CL7 (Karn et al. 2010); *P. aeruginosa* PCP2 (Sharma and Thakur 2008); *P. mendocina* NSYSU (Kao et al. 2004, 2005); *P. veronii* PH-05 (Nam et al. 2003); *P. fluorescens* IST 103 (Thakur et al. 2001, 2002; Shah and Thakur 2003); *Pseudomonas* sp. Bu34 (Lee et al. 1998); *P. aeruginosa* (Premalatha and Rajakumar 1994); *Pseudomonas* sp. strain SR3 (Resnick and Chapman 1994) and *Pseudomonas* sp. strain RA2 (Radehaus and Schmidt 1992). However, these studies need more investigations through structural identification and toxicity assessment of degradation products. Therefore, the characterization of more resistant/degrading bacterial strains will improve our understanding of the metabolic processes involved in the biodegradation of these toxic compounds and could increase the flexibility of the development of effective strategies for managing pesticide risk (Singh 2010; Singh and McDonald 2010).

The present study aimed to isolate and identify OPP and OCP-tolerant bacterial strains, from an artichoke farm soil. In order to provide a more comprehensive knowledge for the future use of bacterial isolates in pesticide bioremediation, the structures of PCP dechlorination products were determined by GC–MS/MS for *P. putida* S148, and the reaction mechanism was elucidated. The biological acute toxicity of the PCP dechlorination products was evaluated using the Toxicity Estimation Software Tool. This study has practical significance for understanding the transformation of PCP by *P. putida* S148. This promising strain could be ecofriendly

used for bioremediation of agricultural soil contaminated with high levels of synthetic pesticides.

Materials and methods

Chemical and reagents

Dimethoate (Dte, MW 229.26 g/mol and > 99% purity) and PCP (MW 226.34 and > 99% purity) were obtained from Sigma-Aldrich (USA). All other chemicals were of the highest purity commercially available.

Soil sampling

Soil samples used in this study were collected from an artichoke field located in the region of Sidi Thabet, (36°54'31.1"N; 10°2'32.89"E), northern Tunisia (in December 2012) during the growing season. This site was highly exposed to different types of pesticides from agricultural activities located in the region. The soil samples were taken at the two depths 0–20 and 20–40 cm, respectively. Systematic sampling of the soil was carried out according to the mixed X modality by a manual auger; this modality was chosen according to the topography of the terrain. The samples were transferred to sterile plastic bags and stored at 4 °C until analysis.

Acclimation, enrichment procedure and isolation of bacteria

The purpose of the acclimation step was to adapt the soil microflora to the pesticide (Cycon et al. 2009). 200 g of soil samples were initially passed through a sieve (4 mm), to remove large pieces of vegetation and debris. Subsequently, samples were supplemented with 200 mg/kg soil of Dte and then incubated in the dark at 30 °C for 90 days. Every 15 days of incubation, the soil was contaminated again with the same concentration of pesticide.

To isolate bacteria capable of tolerating Dte, two enrichment techniques of soil were used. About 10 g of acclimated soil was added to 90 mL of sterile mineral salt medium (MSM), which contained [(NH₄)₂SO₄, 2 g/L; MgSO₄ 7H₂O, 0.2 g/L; CaCl₂ 2H₂O, 0.01 g/L; FeSO₄ 7H₂O, 0.001 g/L; Na₂HPO₄ 12H₂O, 1.5 g/L; KH₂PO₄, 1.5 g/L; pH 8], in 250-mL Erlenmeyer flask supplemented with analytical grade Dte as a sole carbon and energy source. The insecticide was introduced in a form of methanol solution, which was sterilized by filtration (0.2 µm) and added to autoclaved media. After mixing, the culture was incubated at 30 °C on a rotary shaker at 120 rpm for a period of 3 weeks and kept from the light to avoid photo-degradation of Dte. The enriched culture was transferred to fresh MSM every 7 days with

increasing concentrations of pesticide (50–200 mg/L). In the second enrichment technique, 10 g of soil sample was dispersed in 90 mL of MSM (pH 8) containing 50 mg/L of Dte and incubated in the same condition for 3 days. Isolation of pure bacterial strains from the final enrichment cultures was performed by using serial dilution onto MSM agar plates supplemented with 100 mg/L Dte as the sole carbon and energy source. Based on morphological characteristics of the colony, different bacterial strains were selected and purified by repeated streaking on the same medium. The isolates were identified using 16S rRNA molecular analysis.

Taxonomic identification of bacterial isolates

Total genomic DNA was extracted from the pure bacterial strains by sodium dodecyl sulphate (SDS)-proteinase K treatment (Ettoumi et al. 2010). Amplification of the 16S–23S ITS region and the 16S rRNA gene was performed as described by Cherif et al. (2003), respectively. All the PCR products (ITS and 16S rRNA amplicons) were confirmed by electrophoresis, respectively, on 1.2 and 1.5% agarose gels in 0.5× Tris–borate–EDTA buffer and stained for 30 min in 0.5 mg/L ethidium bromide solution.

The amplified 16S rRNA fragments were sequenced and compared with sequences available deposited in the GenBank at the national center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) using the BLAST program (Altschul et al. 1990). A phylogenetic dendrogram was constructed using the neighbor joining method and tree topology was evaluated by bootstrap analysis of 1000 data sets using MEGA 6 (Kumar et al. 2008). The sequences reported in this study have been submitted to NCBI GenBank and the accession numbers are listed in Table 1. BOX-PCR, a technique widely used for typing *Pseudomonas* isolates (Dawson et al. 2002), was performed using the BOX-A1R primer as reported by Cherif et al. (2003).

Pesticide tolerance of bacterial isolates

Pesticide tolerance of the isolates was assayed on MSM liquid medium amended with varying concentrations of Dte and PCP (0.1–5 g/L). After incubation in the same condition above (at 30 °C on a rotary shaker at 150 rpm for 72 h and kept from the light) the optical density was measured (OD_{600}) for screening of most efficient bacterial isolate likely to be employed for further study on biodegradation of PCP.

PCP-degrading bacteria selection

For the selection of PCP-metabolizing bacteria, PCP degradation assay was performed by inoculating each bacterial strain at 1% of inoculum (10^6 CFU/mL) in a 100-mL glass

bottle, containing 40 mL of sterile MSM (pH 7.0 ± 0.2), added with 100 mg/L of PCP as a sole carbon and energy source. The flasks were incubated in a shaker at 30 °C and 150 rpm for up to 168 h. An abiotic control was conducted with uninoculated medium under the same conditions. Quantitative assessment of bacterial growth was assessed by measuring the optical density at 600 nm (OD_{600}). The PCP degradation was quantified every 24 h from 2 mL culture samples using a reverse phase high-performance liquid chromatography (HPLC). HPLC analysis was carried out on a Dionex Ultimate 3000 System fitted on Symmetry C18 column (250×4.6 mm; Inertsil ODS-4, GL Sciences, Japan) and detector UV at 205 nm. The cell suspension was centrifuged, at 8000 rpm for 5 min, and the supernatant was filtered through a 0.22- μ m syringe filter. The column was eluted in an isocratic mode using acetonitrile–water mixture of 50:50 (v/v) as the mobile phase at a flow rate of 1 mL/min. The Chromeleon Software System provided full integration of the readings. The PCP removal was calculated by comparing the peak area obtained from the culture extracts and the area of known quantities of the internal standard (PCP prepared in methanol) (Karn et al. 2010).

Determination of residual PCP and its biodegradation products by GC–MS analysis

For GC–MS analysis, the supernatant was initially extracted three times using dichloromethane in 1:1 ratio. Water was removed from the organic layer by drying with anhydrous sodium sulphate. The solvent was removed under vacuum and subsequently resuspended in 1 mL methanol. Residual PCP and its biodegradation products were analyzed using an “Agilent 7890 B” gas chromatograph system equipped with a “7693” auto-sampler and coupled to a “240-MS” ion trap mass spectrometer (Agilent). The chromatographic separation was performed on a 30-m “Factor four HP-5-M” (5% phenyl methyl polysiloxane) capillary column (internal diameter: 250 μ m, film thickness: 0.25 μ m) from HP. 1 μ L of each sample in the splitless mode. Helium was used as the carrier gas at a constant flow of 1 mL/min. The injector temperature was set at 280 °C. The capillary column was ramped from an initial temperature of 50 °C, held for 5 min, at 10°C/min up to 300 °C where it was held for 1 min. The total duration of GC analysis was 31.0 min. The manifold, ion trap electrodes and transfer line temperatures were set at 50, 220 and 280 °C, respectively. The acquisition was performed in full scan mode. Electron impact (EI) mode was used for this analysis. The instrument was automatically tuned using the m/z ions resulting from EI ionization of perfluorotributylamine (FC-43). In EI, the ionization energy was 70 eV. The mass range was from m/z 50 to m/z 1000, with a scan rate of 3 μ scan/s (Souissi et al. 2013).

Table 1 Identification of pesticide-tolerant bacteria isolated from pesticide-contaminated agricultural soil

Isolates	Enrichment technique ^a	ITS haplotype	Accession number	Closest relative/accession number/blast date	Sequence similarity (%)	Length (bp)	Families
S11	2	H1	KY492719	<i>Pseudomonas putida</i> /NR_074596.1/06-Mar-16	99	769	Pseudomonadaceae
S22	2	H2	KY492714	<i>Pseudomonas putida</i> /NR_074596.1/06-Mar-16	100	650	
S14	2	H2	KY492722	<i>Pseudomonas putida</i> /NR_074596.1/09-Mar-16	99	600	
S16	2	H5	KY492715	<i>Pseudomonas putida</i> /NR_074596.1/06-Mar-16	99	600	
S17	2	H5	KY492720	<i>Pseudomonas putida</i> /NR_074596.1/06-Mar-16	100	630	
S112	2	H10	KY492692	<i>Pseudomonas putida</i> /NR_074739.1/06-Mar-16	99	604	
S113	2	H11	KY492698	<i>Pseudomonas putida</i> /NR_074739.1/07-Mar-16	99	730	
S114	2	H10	KY492693	<i>Pseudomonas putida</i> /NR_074739.1/06-Mar-16	99	601	
S116	2	H13	KY492721	<i>Pseudomonas putida</i> /NR_074596.1/09-Mar-16	99	650	
S118	2	H10	KY492699	<i>Pseudomonas putida</i> /NR_074739.1/08-Mar-16	99	700	
S119	2	H10	KY492703	<i>Pseudomonas putida</i> /NR_074739.1/08-Mar-16	100	650	
S220	2	H14	KY492694	<i>Pseudomonas putida</i> /NR_074739.1/06-Mar-16	99	603	
S121	2	H10	KY492695	<i>Pseudomonas putida</i> /NR_074739.1/06-Mar-16	99	558	
S122	2	H15	KY492716	<i>Pseudomonas putida</i> /NR_074596.1/06-Mar-16	99	600	
S123	2	H15	KY492717	<i>Pseudomonas putida</i> /NR_074596.1/06-Mar-16	99	600	
S125	2	H15	KY492723	<i>Pseudomonas putida</i> /NR_074596.1/09-Mar-16	99	650	
S126	2	H17	KY492704	<i>Pseudomonas putida</i> /NR_074739.1/08-Mar-16	99	750	
S127	2	H15	KY492718	<i>Pseudomonas putida</i> /NR_074596.1/06-Mar-16	99	755	
S228	2	H18	KY492700	<i>Pseudomonas putida</i> /NR_074739.1/08-Mar-16	99	700	
S230	2	H20	KY492697	<i>Pseudomonas putida</i> /NR_074739.1/06-Mar-16	99	600	
S232	2	H15	KY492701	<i>Pseudomonas putida</i> /NR_074739.1/08-Mar-16	99	700	
S233	2	H22	KY492702	<i>Pseudomonas putida</i> /NR_074739.1/08-Mar-16	99	700	
S134	2	H15	KY492724	<i>Pseudomonas putida</i> /NR_074596.1/09-Mar-16	99	700	
S136	1	H23	KY492705	<i>Pseudomonas putida</i> /NR_074739.1/06-Mar-16	100	650	
S139	1	H23	KY492711	<i>Pseudomonas putida</i> /NR_074739.1/09-Mar-16	99	700	
S140	1	H23	KY492709	<i>Pseudomonas putida</i> /NR_074739.1/09-Mar-16	99	700	

Table 1 (continued)

Isolates	Enrichment technique ^a	ITS haplotype	Accession number	Closest relative/accession number/blast date	Sequence similarity (%)	Length (bp)	Families
S145	1	H23	KY492712	<i>Pseudomonas putida</i> /NR_074739.1/11-Mar-16	99	700	
S148	1	H23	KY492706	<i>Pseudomonas putida</i> /NR_074739.1/11-Mar-16	99	700	
S249	1	H26	KY492708	<i>Pseudomonas putida</i> /NR_074739.1/11-Mar-16	99	700	
S257	1	H27	KY492710	<i>Pseudomonas putida</i> /NR_074739.1/11-Mar-16	99	700	
S260	1	H27	KY492707	<i>Pseudomonas putida</i> /NR_074739.1/11-Mar-16	99	600	
S28	2	H6	KY492726	<i>Pseudomonas brenneri</i> /NR_025103.1/07-Mar-16	99	700	
S211	2	H9	KY492729	<i>Pseudomonas brassicacearum</i> /NR_074834.1/08-Mar-16	99	750	
S115	2	H12	KY492727	<i>Pseudomonas fuscovaginae</i> /NR_116700.1/08-Mar-16	99	700	
S143	1	H24	MF495494	<i>Pseudomonas</i> sp./MF948932.1/01-Oct-2017	99	600	
S146	1	H25	MF495495	<i>Pseudomonas</i> sp./DQ256393.1/01-Oct-2017	99	689	
S13	2	H3	KY492725	<i>Acinetobacter calcoaceticus</i> /NR_117619.1/07-Mar-16	99	750	Moraxellaceae
S25	2	H4	MF495498	<i>Bacillus cereus</i> /NR_074540.1/17-Jul-17	99	659	Bacillaceae
S29	2	H7	KY492689	<i>Bacillus pumilus</i> /NR_074977.1/07-Mar-16	100	600	
S110	2	H8	KY492690	<i>Bacillus oceanisediminis</i> /NR_117285.1/07-Mar-16	99	603	
S264	1	H31	KY492691	<i>Bacillus oceanisediminis</i> /NR_117285.1/07-Mar-16	100	750	
S256	1	H28	MF495497	<i>Bacillus flexus</i> /NR_113800.1/17-Jul-17	99	581	
S124	2	H16	KY492713	<i>Flavobacterium anhuiense</i> /NR_044388.1/07-Mar-16	99	598	Flavobacteriaceae
S229	2	H19	MF373464	<i>Comamonas aquatica</i> /NR_042131.1/07-Mar-16	99	600	Comamonadaceae
S231	2	H21	KY492728	<i>Achromobacter spanius</i> /NR_025686.1/08-Mar-16	100	750	Alcaligenaceae
S262	1	H29	KY492730	<i>Rhodococcus ruber</i> /NR_118602.1/11-Mar-16	99	750	Nocardaceae
S263	1	H30	KY492732	<i>Ochrobactrum anthropi</i> /NR_074243.1/09-Mar-16	100	700	Brucellaceae
S268	1	H30	KY492733	<i>Ochrobactrum anthropi</i> /NR_074243.1/09-Mar-16	99	680	
S265	1	H32	KY492734	<i>Aquamicrobium aestuarii</i> /NR_108709.1/09-Mar-16	98	700	Phyllobacteriaceae
S266	1	H33	KY492735	<i>Bordetella petrii</i> /NR_074291.1/06-Mar-16	99	600	Alcaligenaceae
S267	1	H34	KY492731	<i>Microbacterium saccharophilum</i> /NR_114342.1/06-Mar-16	99	699	Microbacteriaceae

^aFirst enrichment technique (1); second enrichment technique (2)

In silico toxicity estimation using Toxicity Estimation Software Tool

For toxicity assessments, the Toxicity Estimation Software Tool (T.E.S.T.) software was used (Martin et al. 2016). It is an online available computerized predictive system (<http://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test>) developed by the US Environmental Protection Agency to easily estimate toxicity using a variety of Quantitative Structure Activity Relationship (QSAR) mathematical models. Toxicity values were estimated averaging the predicted toxicities, to provide that the predictions were within the respective applicability domains of the QSAR models.

Results and discussion

Two enrichment techniques were adopted to isolate pesticide-tolerant bacteria from pesticide-contaminated agricultural soil sampled during the growing season of artichoke. A collection of 68 strains was obtained including 35 strains recovered from soil samples already contaminated by pesticides and 33 strains from acclimated soil samples. ITS-PCR fingerprinting was applied as a first molecular technique to assess bacterial diversity of the collection. The obtained results showed a significant bacterial diversity with the detection of 34 distinct haplotypes (Fig. 1). This bacterial diversity was differentially expressed and related to the enrichment techniques used for bacterial isolation. Thus, the first approach showed 12 haplotypes versus 22 other totally different haplotypes with the second one (Table 1). The ITS-PCR profiles contained 1–11 reproducible bands with sizes ranging from 100 to about 2000 bp. H23 and H27

Fig. 1 Dereplication of the collection based on the amplified internal transcribed spacers 16S-23S rRNA: ITS-PCR analysis. **a** Haplotypes 1–22, obtained with the acclimated soil (the second enrichment technique); **b** haplotypes 23–34 obtained with soil already contaminated (the first enrichment technique); M: molecular size marker (100 bp + 2 kb + 3 kb), isolates: S11; S22; S13; S14; S25; S16; S17;

S28; S29; S110; S211; S112; S113; S114; S115; S116; S117; S118; S119; S220; S121; S122; S123; S124; S125; S126; S127; S228; S229; S230; S231; S232; S233; S134; S135; S136; S137; S138; S139; S140; S141; S142; S143; S144; S145; S146; S147; S148; S249; S250; S251; S252; S253; S254; S255; S256; S257; S258; S259; S260; S261; S262; S263; S264; S265; S266; S267; S268

represent the most encountered haplotypes revealed in 10 and 11 strains isolated from acclimated soil samples (first enrichment approach), respectively (Fig. 1b). However, the second most frequent represented patterns were recovered from soil samples already contaminated with pesticide, presented by haplotypes H10 (6 strains) and H15 (7 strains) (Fig. 1a). The remaining ITS haplotypes were found in one or two isolates. Overall, the use of both techniques might be helpful to get a deep insight into the bacterial diversity.

Sequencing of partial 16S rRNA gene was carried out for representative bacterial isolates of each distinct haplotype ($n=51$) and was analyzed by BLAST algorithm (Table 1). Phylogenetic analysis revealed that the isolates were allocated into 11 different genera with an uneven distribution *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, *Comamonas*, *Achromobacter*, *Rhodococcus*, *Ochrobactrum*, *Aquamicrobium*, *Bordetella*, *Microbacterium* (Fig. 2a). *Pseudomonas* was the most abundant genus ($n=36$), exhibiting 99–100% identity to published species sequences. *Pseudomonas* strains were represented by 22 ITS types and were divided in five species, among which one species was the most recovered during the isolation steps: *P. putida* with 19 different ITS types. All isolates were placed into four major bacteria phyla Firmicutes (9.80%), Actinobacteria (3.92%), Bacteroidetes (1.96%) and Proteobacteria. This later was the most dominant with 84.31% of isolates and was represented by three subclasses, the Alphaproteobacteria, Betaproteobacteria and the most abundant Gammaproteobacteria (72.54%). Our results were in agreement with other studies which reported that these bacteria are effective indigenous microorganisms in remediation of pesticide-polluted soils and could be highly active micro-degraders (Doolotkeldieva et al. 2017). Moreover, Parte et al. (2017) and El-Bestawy et al. (2000) extensively proved the marvelous superior potentiality of *Pseudomonas* for biodegradation of toxic organic pollutants. In our study, the dominance of this genus through two enrichment techniques of soil samples confirmed and reflected its high resistance to pesticides.

Our results highlight that 16S rRNA-based phylogenetic analysis is limited for identifying closely related species or for detecting intraspecific diversity. For resolving the genetic heterogeneity of *P. putida* population, BOX-PCR was applied to the 31 isolates. This technique allows a high degree of discrimination between strains. The gel electrophoresis of resulting PCR products showed 1–9 reproducible bands with different intensities varying from 180 to 1400 bp (Fig. 3). According to the obtained BOX-PCR dendrogram, 22 distinct profiles were acquired enclosing two major clusters, versus 19 ITS haplotypes, confirming the microdiversity of pesticide-tolerant *P. putida*. Among them, a single strain (*P. putida* S148) was able to grow and exhibited a high tolerance to Dte (4 g/L) and PCP (1 g/L) (Fig. 2b), while others can tolerate a Dte concentration ranging from 1 to

3.5 g/L and from 0.1 to 1 g/L for PCP. This high-tolerant isolate, *P. putida* S148 was selected for further biodegradation capability research.

PCP degradation supported cell growth, indicating that S148 strain could use PCP as a carbon source (Fig. 4a, b). According to HPLC analysis, S148 showed a maximum PCP removal of about 91% after 168 h of incubation on liquid MSM medium supplemented with 100 mg/L of PCP (Fig. 4c). To evaluate the effect of adsorption, the experiments were repeated with heat-killed cells. Heat-killed S148 did not have any effect on the degradation of PCP, suggesting that the decrease was not due to adsorption. Similar studies, showed that aerobic bacteria particularly strains of *Pseudomonas* genus, were able to utilize PCP compounds as the sole carbon and energy source. Shah and Thakur (2003) demonstrated that *P. fluorescens* was able to degrade 72% PCP in 96 h of incubation in the growth medium supplemented with 100 mg/L of PCP. Karn et al. (2010) observed 66.8% PCP removal by *Pseudomonas stutzeri* strain CL7 when grown for 2 weeks with 100 mg/L PCP. Chen et al. (2013) reported that *P. putida* was able to remove 57.2% of PCP within 40 h when grown with 100 mg/L of PCP. Both aerobic and anaerobic bacteria are able to degrade PCP but aerobic degradation processes are generally preferred. Aerobic bacteria (particularly pseudomonads), are more efficient for degrading toxic compounds, because they grow faster and usually achieve complete mineralization of toxic organic compounds to inorganic compounds (Kim et al. 2002; Kargi and Serkan 2004; Ammeri et al. 2017).

Figure 4e, f shows two chromatograms of pentachlorophenol (PCP) before and after the biodegradation using *P. putida* S148. The appearance of numerous biodegradation products can clearly be distinguished compared to the reference solution. The first step of this study is to analyze the spectrum of the reference compound to identify the characteristic losses signing the identity of the original compound. Thus, the spectrum indicates the presence of the chlorine atom with the difference of two units between the peaks $M+2$ m/z 264, m/z 266, m/z 268 in m/z 270. Also, the presence of aromatic ring in its structure giving a great stability to the molecule can explain the patterns of intensity of molecular ions (de Oliveira et al. 2011).

Pentachlorophenol (PCP) and other chlorophenols (CPs) are known to be very toxic compounds. Thus, degradation of those PCP and other CPs in environmental matrices represents an important natural removal route. PCP and CPs degradation may occur through several routes in the environment including chemical, microbiological photochemical or thermal processes. Among the most important decomposition pathways of PCPs, microbial one was recognized as very important. Some bacterial strains were able to use PCP and its metabolite as carbon and energy sources (Arora and Bae 2014). Several strains of *Pseudomonas*, including *P.*

Fig. 2 **a** Phylogenetic analyses of bacterial isolates based on 16S rRNA partial sequences. Phylogenetic dendrogram was evaluated by performing bootstrap analysis of 1000 data sets using MEGA 6. 16S rRNA sequence accession numbers of the reference strains are indicated in the brackets. **b** Maximum tolerated concentration of pesticide (Dte/PCP) in liquid MSM (48 h of incubation/30 °C/150 rpm/in the dark)

fluorescens (Shah and Thakur 2003), *P. aeruginosa* (Sharma and Thakur 2008) and *P. stutzeri* (Karn et al. 2010) were reported to be interesting examples. In this study, *P. putida*

S148 was also proven to be endowed with PCP biodegradation potential. Nevertheless, mastering the results of those biodegradation reactions is however a limiting and decisive

Fig. 3 Cluster analyses of BOX-PCR fingerprints showing the genotypic diversity of 31 isolates of *P. putida*. The dendrogram was obtained from similarity coefficient (Dice) calculations and clustering was done using unweighted pair-grouping method based on arithmetic averages (UPGMA) algorithm using InfoQuest FP software. The dendrogram resulted in 2 major clusters and 22 distinct BOX profiles

step. Pentachlorophenol biodegradation products analysis was made through the investigation of the peaks shown in Fig. 4f. The main identified PCP biodegradation products are represented in Table 2.

Among the recovered biotransformation products, tetrachlorophenol, trichlorophenol, dichlorophenol and chlorophenol were identified. The most stable and abundant one was the tetrachlorophenol. The detection of ions at m/z 232 at two retention times indicates that the chlorine atom losses leading to the formation of the tetrachlorophenol may occur at several positions. Table 2 indicates the spectrum of the identified 2,3,4,6-tetrachlorophenol and 2,3,5,6 tetrachlorophenol. Nam et al. (2003) studied the PCP transformation ability of a bacterial strain, *P. veronii* PH-05, isolated from a timber storage yard by selective soil enrichments and

able to grow using PCP as a sole carbon and energy source. As identified in our study, they also reported the detection of tetrachlorophenol as a metabolic intermediate. However, they proved that this strain PH-05 can metabolize PCP when tetrachlorocatechol is not accumulated, but cannot metabolize PCP after tetrachlorocatechol accumulation. This observation should be taken into consideration in our study as this compound was detected in a large proportion. Among the other main detected biodegradation compounds, tetrachlorohydroquinone was well distinguished. The generation of this biotransformation product was already reported in microbial PCP degradation studies. Dichlorohydroquinone, trichlorohydroquinone were identified at a very weak trace amount. Accordingly, those compounds would probably be intermediate metabolites. In fact, tetrachloro-*p*-hydroquinone,

Fig. 4 **a** Petri plate showing *P. putida* S148 grown in solid medium. **b** Growth of *P. putida* S148 in MSM supplemented with 100 mg/L of PCP as a sole carbon and energy source (30 °C, pH 7.0 ± 0.2 and 150 rpm). **c** HPLC chromatogram of PCP degradation by *P. putida* S148 compared with control after 168 h incubation period. **d** Growth

curve of S148 and PCP degradation in MSM containing 100 mg/L of PCP. GC-MS chromatogram of PCP degradation by *P. putida* S148 (KY492706) compared with control (e) after 168 h incubation (f). All the experiments in this study were performed in triplicate and values are mean of three replicates

2,4,6-trichlorophenol, and 2,6-dichlorophenol as PCP degradation products were also recently reported in Garg et al. (2013) study dealing with a novel psychrotrophic *P. putida* SKG-1 strain tested for simultaneous bioremediation of pentachlorophenol and hexavalent chromium.

Arora and Bae (2014) highlighted that for *Sphingomonas chlorophenolicum*, PCP degradation was initiated by the formation of tetrachlorohydroquinone after hydroxylation at the

para-position using PCP-4-monooxygenase or cytochrome P-450 type enzyme. PCP-4-monooxygenase catalyzes the conversion of PCP to tetrachlorohydroquinone through the removal of chloride ions (Arora and Bae 2014).

Trace amounts of pentachloroanisole were also detected in this study (Table 2). This transformation product is probably formed through the methylation of the PCP. It is a more lipid soluble compound. No literature data nor degradation

Table 2 Major PCP biodegradation products from *P. putida* S148, identified using GC–MS analysis

Compound	Retention time (min)	Structure	Molecular formula	MW	Selected fragments m/z
PCP	16,516		C ₆ HCl ₅ O	266	230, 202, 165
Tetrachlorophenol	14,198		C ₆ HCl ₄ O	232	194, 166, 131
	18,13	Phenol, 2,3,4,6 tetrachloro	C ₆ HCl ₄ O	232	194, 166, 131
Trichlorophenol	16,03		C ₆ H ₃ Cl ₃ O	197	160, 132, 97
Dichlorophenol	13,54		C ₆ H ₄ Cl ₂ O	162	126, 98, 63
Chlorophenol	9,27		C ₆ H ₅ ClO	128	100, 65, 39
Tetrachlorohydroquinone (TCHQ)	17,29		C ₆ H ₂ Cl ₄ O ₂	248	212, 182, 175
Pentachloroanisol	17,04		C ₇ H ₃ Cl ₅ O	280	265, 237, 165

pathway of PCP describing this product were previously reported in the literature. Further experiments should be performed to confirm its presence.

Aiming to assess whether *P. putida* S148 would be an effective and promising strain for PCP bioremediation, it is crucial to investigate if the produced metabolites are endowed with a similar toxic effect as the one reported for the parent compound. In fact, in addition to its stability and persistency, PCP was reported as an important pesticide from a toxicological point of view. Therefore, a specific interest was shown toward the major identified compounds,

the tetrachlorophenol (2,3,4,6 tetrachlorophenol and 2,3,5,6 tetrachlorophenol) and the tetrachlorohydroquinone. The other metabolites, detected at fewer concentrations could be considered as intermediate compounds. A kinetic study should be performed to confirm this hypothesis. As a preliminary toxicological study of PCP biodegradation products, T.E.S.T described in the experimental part, was used to study the effect of PCP and the three selected metabolites on fathead minnow (*Pimephales promelas*), *Daphnia magna* and *Tetrahymena pyriformis*. Toxicity estimation was also performed using developmental toxicity assay.

Fig. 5 In silico estimated toxicities for PCP and its main biodegradation products [*Fathead minnow* LC50 (96 h), *Daphnia magna* LC50 (48 h) and *T. pyriformis* IGC50 (48 h)]

96 h *P. promelas* 50% lethal concentration (LC50), 48 h *D. magna* 50% lethal concentration (LC50) and *T. pyriformis* 50% growth inhibition concentration (IGC50) were first estimated. As shown in Fig. 5, the toxicity estimation using those organisms showed that the LC50 and IGC50 obtained values for the three metabolites were slightly higher than those estimated for the PCP indicating that the metabolites are slightly less toxic than the original compound for those specific organisms. Similar results were previously reported for PCP and 2,3,4,6 tetrachlorophenol as it was demonstrated, using acute *Daphnia* tests, that Phenol, 2,3,4,6 tetrachloro was less toxic to *Daphnias* than PCP (Liber and Solomon 1994). Another study (Devillers and Chambon 1986) showed that both 2,3,4,5 tetrachlorophenol and 2,3,5,6 tetrachlorophenol were less toxic than PCP in 24 h acute experiments with *D. magna*.

Conclusion

Bacterial strains isolated from pesticide-contaminated agricultural soil in Tunisia, able to grow on the presence of high concentrations of organochlorine and organophosphate pesticides were studied. Pesticide-tolerant bacterial strains were subjected to identification, characterization and phylogenetic analysis. The majority of bacterial isolates belonged to *Pseudomonas* genus. The selected strain, *P. putida* S148 was able to degrade PCP and various degradation products (tetrachlorophenol, trichlorophenol, dichlorophenol and

chlorophenol) were detected in the minimal medium during bacterial growth. Further in situ bioremediation experiments using pesticide-contaminated soils inoculated with a single *Pseudomonas* strain or a consortium are in progress.

Acknowledgements The authors thank the financial support of the South Africa/Tunisia-research partnership programme bilateral agreement (ADMEN project) and the Tunisian Ministry of Higher Education and Scientific Research in frame of the Laboratory project LR11ES31.

Compliance with ethical standards

Conflict of interest We declare that there is no conflict of interest regarding the publication of this article.

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