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Chlorpyrifos biodegradation and 3,5,6-trichloro-2-pyridinol production by actinobacteria isolated from soil

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ABSTRACT

Chlorpyrifos (CP) is a widely used agricultural insecticide that is hazardous to both the environment and human health. Therefore, it is an essential to develop approaches to remove this compound from contaminated soils, water and sediments. In this study, actinobacteria were isolated from an agricultural soil that had received continuous applications of CP. Four strains were selected as a result of their tolerance to 50 mg L^{-1} of CP in agar plate and they were identified as *Streptomyces* sp. based on 16S rDNA. According to relationship of CP degradation and microbial growth studies, two isolates were selected and were named *Streptomyces* sp. strain AC5 and *Streptomyces* sp. strain AC7. The strains were cultivated in liquid medium with CP at concentrations of 25 mg L^{-1} and 50 mg L^{-1} for 72 h. The results indicated that both strains were able to rapidly degrade CP with about 90% degradation after 24 h of incubation. A different pattern of CP degradation was observed when its main metabolite, 3,5,6-trichloro-2-pyridinol (TCP) was monitored. A maximum concentration of 0.46 mg L^{-1} of TCP was produced by *Streptomyces* sp. strain AC5 and its concentration decreased as a function of time. In contrast, TCP production by *Streptomyces* sp. AC7 increased over time from 1.31 mg L^{-1} to 4.32 mg L^{-1}. CP degradation was associated to microbial growth of the strains, pH modification, glucose consumption and organic acids excretion in the liquid medium. This work constitutes one of the few reports of *Streptomyces* as CP-degraders. Given the high CP degradation observed here, the *Streptomycetes* strains show a good potential as CP-degrading actinobacteria.

1. Introduction

Organophosphorus pesticides (OPs) are the most widely used group of pesticides, accounting for more than 36% of the total world market (Kanekar et al., 2004; Singh and Walker, 2006). Among OPs, CP (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) has been on the market for over 40 years and today is one of the most widely used pest control products of significant crops in diverse countries. In Chile, CP is extensively used in the fruit farming industry, especially in blueberry crops. In recent years, this crop has been characterized by high productivity due to planted areas that have increased from 1910 ha in 2001 (INDAP, 2002) to 10,763 ha in 2008 (Rosas, 2009) today this crop would reach 12,500 ha. This has meant an increase in the use of CP, most likely increasing the risk of environmental contamination and health hazards to consumers by CP residues that have accumulated on agricultural crops (Kim and Ahn, 2009; Singh et al., 2011). The contamination of soil by CP can be caused from handling the pesticide in the farmyard and from the rinsing of containers. Moreover, accidental releases can result in the contamination of surface and groundwater (Singh et al., 2004).

The insecticide CP is characterized as moderately persistent with a half-life in soil of 10–120 days, and it has very low solubility in water (2 mg L^{-1}). In soil, the degradation of CP can involve a combination of photolysis, chemical hydrolysis and microbial degradation. Biodegradation is considered a viable and environmentally friendly approach to the decontamination of CP. A review by Singh and Walker (2006) explained how bacteria and fungi degrade CP through catabolic and co-metabolic mechanisms. In most cases, aerobic bacteria tend to hydrolyze CP to produce diethylthiophosphoric acid and 3,5,6-trichloro-2-pyridinol (TCP),

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the latter of which is the primary and major degradation product of CP (Xu et al., 2007). The accumulation of TCP, which has antimicrobial properties, has been attributed to the prevention of the proliferation of CP-degrading microorganisms (Singh and Walker, 2006). However, studies have consistently shown that CP and TCP can both be degraded efficiently by bacteria isolated from contaminated soils (Yang et al., 2005; Li et al., 2008; Lakshmi et al., 2009). Examples of CP and TCP-degrading bacteria include *Alcaligenes faecali*, *Bacillus pumilis* and *Pseudomonas aeruginosa* (Yang et al., 2005; Li et al., 2008; Anwar et al., 2009; Lakshmi et al., 2009). Studies on microbial degradation are useful for the development of bioremediation strategies for the decontamination of pesticides using microorganisms that are typically present in contaminated soils (Fantroussi and Agathos, 2005; Cycón et al., 2009).

Actinobacteria, a group of bacteria that were formerly classified as actinomycetes, constitute a significant fraction of the microbial population in soils (commonly more than 1 million microorganisms per gram) (Goodfellow and Williams, 1983; Seong et al., 2001). These microorganisms have been recognized as having great potential for the biodegradation of certain organic compounds, such as pesticides (Castillo et al., 2006). A review by De Schrijver and De Mot (1999) indicates that degradation of organochlorines, s-triazines, carbamates, acetanilides, organophosphorus and sulfonylurea is predominantly attributed to actinobacteria. Recent research has also shown that, specifically, the actinobacteria of genus *Streptomyces* have the ability to degrade pesticide such as alachlor (Sette et al., 2005), diuron (Castillo et al., 2006), lindane, chlordane, methoxychlor (Benimeli et al., 2007; Fuentes et al., 2010) and cypermethin (Lin et al., 2011). Little information about the degradation of CP by actinobacteria has been reported. In addition, these bacteria may be well suited for inoculation in decontamination systems of pesticides as a consequence of their ability to colonize the soil. This is facilitated through their growth as vegetative hyphal masses that can differentiate into spores, assisting in their spreading and persistence. The spores represent a semi-dormant stage in the life cycle of the bacteria, and the spores can survive in soil for long periods of time in conditions of low nutrients and low water availability (McCarthy and Williams, 1992).

Despite their interesting biotechnological characteristics, relatively few actinobacteria have been evaluated for their ability to degrade pesticides, specifically of the organophosphate class. In this context, new methods are needed to detect, isolate and characterize the strains that play a role in the degradation process of pesticides. The aim of this work was to isolate and characterize CP-degrading actinobacteria that were isolated from agricultural soil with a history of CP applications, and to evaluate their abilities to degrade CP and to produce the main degradation by-product, the metabolite TCP.

2. Methodology

2.1. Sampling

Soil samples were collected from a blueberry field that was located in the city of Gorbea in southern Chile (39° 06′ 11″ S; 72° 40′ 48″ W). The soil has been defined as an Andisol (Typic Hapludands) (CIREN, 2003). This soil had been exposed to continuous applications of CP for a considerable period of time. Surface soil samples (pH 5.2, 15.6% organic matter, 18.62 mg kg^{-1} nitrogen and 16.08 mg kg^{-1}phosphorus) were collected adjacent to blueberry trees, air-dried, sieved through a 2 mm mesh and stored at 4 °C until their use.

2.2. Isolation of actinobacteria

Ten grams of the soil sample was added to sterilized distilled water (90 mL), and the suspension was shaken vigorously (150 rpm) at room temperature (20 °C) for 2 h. Serial dilutions (10^{-2}–10^{-5}) were prepared using sterilized distilled water, and 0.1 mL aliquots were inoculated in Petri dishes that contained starch casein agar medium (agar, 15.0 g; soluble starch, 10.0 g; K_2HPO_4, 2.0 g; KNO_3, 2.0 g; NaCl, 2.0 g; casein, 0.3 g; $MgSO_4 \bullet 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4 \bullet 7H_2O$, 0.01 g; distilled water, 1000 mL), ISP-2 agar medium (agar, 20.0 g; malt extract, 10.0 g; yeast extract, 4.0 g; glucose, 4.0 g; distilled water, 1000 mL) and ISP-5 agar medium (agar, 20.0 g; glycerol, 10.0 g; L-asparagine, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \bullet 7H_2O$, 0.2 g; $FeSO_4 \bullet 7H_2O$, 0.01 g; distilled water, 1000 mL) (Atlas, 1995). The pH of the media was adjusted to 7.0–7.2 with HCl 0.1 M prior to sterilization (121 °C for 20 min). Each medium was supplemented with 10.0 μg mL^{-1} of nalidixic acid and cycloheximide to inhibit the growth of Gram-negative bacteria and fungi, respectively.

Following inoculation, the plates were incubated at 28 °C for up to 10 days, and colonies were purified without antibiotics by streaking onto the respective agar medium from which the colonies were isolated. Microbial selection was based on colony morphology, color and presence of diffusible pigments according to Bergey's manual (Lechevalier, 1989). The actinobacteria strains were maintained on ISP-2 agar medium slants at 4 °C and were filed at the Laboratory of Environmental Biotechnology from Universidad de La Frontera.

2.3. CP-Tolerant actinobacteria

Preliminary qualitative and quantitative screening experiments were performed to obtain strains that were tolerant to CP. Qualitative screening consisted of evaluating the growth of isolated strains in plates containing MM with agar that was inoculated with CP dissolved in methanol (Sigma–Aldrich, 99% pure) at 1, 10, 25 and 50 mg L^{-1}. The plates were incubated at 28 °C for 7 days. After this time, the strains were evaluated as negative and positive growth.

Quantitative screening consisted of evaluating the degradation of 25 mg L^{-1} CP in liquid medium after 72 h by strains that exhibited growth at the highest CP concentration that was assayed in the previous screening. The concentration 25 mg L^{-1} CP was chosen because of CP-tolerant strains had higher growth and development of colonies compared to solid medium with 50 mg L^{-1} CP. The study was conducted as described below. From this experiment, the results of degradation (%) and microbial growth (%) with respect to a control without CP (biotic control) and with CP without bacteria (abiotic control) were used to obtain a selection index (degradation/microbial growth) that was previously proposed by Benimeli et al. (2007) with modifications. Our criterion was to obtain further degradation using the least amount of biomass.

2.4. Characterization of selected CP-tolerant strains

Pure cultures of strains AC5, AC6, AC7 and AC9 were grown in ISP-2 broth for 4 days, and chromosomal DNA was extracted using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Inc., USA). For genetic affiliation analysis, 16S rDNA gene fragments were amplified by PCR according to Pearce et al. (1994) using the universal bacterial primer set 27f/1492r. The amplified fragments that were obtained were purified and sequenced by Macrogen, Inc., (Korea). The sequencing was performed in both directions (sense and antisense) to provide reliable nucleotide sequences. The consensus nucleotide sequences that were obtained in this study were deposited and compared with those present in the GenBank database from the National Center for Biotechnology Information (NCBI) using the BLAST tool for alignment and identification (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic affiliation in

relation to representative OPs-degrading bacteria in GenBank was performed using MEGA4 (http://www.megasoftware.net/).

After genetic characterization, the selected strains AC5 and AC7 were subjected to biochemical and enzymatic characterization using API 20E and API CORYNE test strips (Biomerieux®). Moreover, their tolerance to pH (growth in liquid medium) was evaluated in 10 mL of ISP-2 medium that had been inoculated with the respective strain. The incubation was performed at 28 °C with shaking (100 rpm) for 7 days.

2.5. Degradation of CP in liquid medium

Starter cultures of spores and mycelia of strains were grown in 100 mL Erlenmeyer flasks that contained 30 mL of ISP-2 medium. The inoculated flasks were incubated for 96 h at 28 °C and 100 rpm in a rotary shaker. The cultures were centrifuged (8000 × g for 10 min at 4 °C), and the cell pellets were washed with sterile saline solution.

The degradation experiments in liquid media were conducted in 100 mL flasks that contained 30 mL of liquid-defined minimal medium (MM) (glucose, 4.0 g; L-asparagine, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \bullet 7H_2O$, 0.20 g; $FeSO_4 \bullet 7H_2O$, 0.01 g, distilled water, 1000 mL) (Amoroso et al., 2001). CP dissolved in methanol was added to the flasks at concentrations of 25 mg L^{-1} and 50 mg L^{-1}. Then, samples of cell pellet were added to a final concentration of 4% (*w*/*v*) wet weight, non-inoculated flasks were run as control. The flasks were then incubated at 28 °C on a rotary shaker (100 rpm). The samples were taken out at 0, 24, 48 and 72 h, and the parameters analyzed were pH, glucose consumption, biomass expressed as dry weight (mg mL^{-1}), the residual concentration of CP and the main CP metabolite, TCP. The biomass obtained after 24 h of incubation was used to determine adsorption to the cell. Additionally, the supernatant was used for the determination of organic acids, which was considered as a complementary analysis for a better understanding of the results.

2.6. Chemical analyzes

Supernatant samples were used to glucose determination using the dinitrosalicylic colorimetric method (Miller, 1959). Briefly, 3 mL of dinitrosalicylic acid reagent solution (1%) were added to 3 mL of sample in a lightly capped test tube. The tubes were heated at 90 °C for 5 min to develop the red–brown color. Then, 1 mL of a 40% potassium sodium tartrate solution was added to stabilize the color. Finally, after cooling to room temperature in a cold water bath, the absorbance was measurement with a spectrophotometer at 575 nm.

Supernatant samples of the centrifuged cultures were used to determine residual CP by gas chromatography (GC-NPD). Liquid samples (1 mL) were diluted to a volume of 10 mL with distilled water and were extracted (2×) with 10 mL of hexane by vortex mixing (2×, 10 s) and then by shaking on a rotary shaker for 10 min at 200 rpm. The organic extracts were combined and dehydrated with Na_2SO_4. Then, the samples were stored at −20 °C for chromatographic analysis. The analyses were performed using a Shimadzu gas chromatograph GC-2014 that was equipped with an RTX-5 capillary column (Crossbond® 5% diphenyl/95% dimethyl polysiloxane, 30 m, 0.32 mm i.d., film thickness 0.25 μm) and NPD detector. The injection and detector temperatures were 280 °C and 300 °C, respectively. The oven temperature program began at 90 °C for 1 min, increased to 180 °C at 15 °C/min, increased to 240 °C at 5 °C/min and then increased to 280 °C at 15 °C/min. The obtained data were analyzed with the program GC Solution Version 2.30.00 (GC Solution Analysis Copyright © 2000–2004 Shimadzu). Under these chromatographic conditions, the retention time for CP was 12.5 min. The calibration curve was linear in the range of 0.01–2.5 mg L^{-1} with $R^2 = 0.99$. The recovery of CP in the liquid medium was 90%.

TCP was analyzed directly in the liquid samples, after filtration through 0.22 μm filter, by HPLC on a LiChrospher® 60 RP-select B column (5 μm) using a Merck–Hitachi instrument that was equipped with an L-7455 diode array detector. The readings were performed at 205 nm with a retention time of 4.6 min. The mobile phase was 0.1 M phosphoric acid-acetonitrile 50/50 (*v*/*v*). The mobile phase flow rate was 1.0 mL min^{-1}, and the injected sample volume was 20 μL. The efficiency of TCP recovery from the liquid medium was 94%.

After 24 h of incubation the biomass was obtained and CP extracted with 10 mL of hexane by vortex mixing for 10 s and then by shaking on a rotary shaker for 10 min at 200 rpm. With these results the adsorption of CP in the cells was obtained.

Organic acids determination was carried out by HPLC (Cawthray, 2003) on a Symmetry C18 column (Waters 5 μm, 250 mm × 4.6 mm), with a Merck–Hitachi instrument model 3400 equipped with a UV detector set at 210 nm. The mobile phase was 93% 25 mM KH_2PO_4 at pH 2.5 and 7% methanol at a flow of 1.0 mL min^{-1}. In total nine low molecular-mass organic acids standards of analytical quality were used. The organic acids analyzed were gluconic, oxalic, malic, pyruvate, lactic, acetic, maleic, citric and succinic. The presence of acids was confirmed by addition of standard into the sample.

2.7. Data analysis

All of the experiments were performed in triplicate. The data were statistically analyzed using one-way analysis of variance (ANOVA). When significant differences were observed, the means were separated using Tukey's minimum significant difference test ($p \leq 0.05$).

3. Results

3.1. Isolation and screening for CP-tolerant strains

In the present study, a total of 30 microorganisms were isolated from agricultural soil that was characterized by repeated application of CP. The strains were obtained in pure culture and were initially screened for microbiological tolerance in solid culture medium with increasing concentrations of CP that ranged from 1 mg L^{-1} to 50 mg L^{-1}. These assays showed that 23 (76%) out of the 30 tested strains grew at 1 mg L^{-1} CP, 12 (40%) strains grew at concentrations of 10 mg L^{-1} and 25 mg L^{-1} and that only 4 (13%) out of the 30 strains grew at a concentration of 50 mg L^{-1}. These last strains were named AC5, AC6, AC7 and AC9.

The four selected strains were screened to determine their growth capacity and their ability to degraded CP in liquid culture medium applied at a concentration of 25 mg L^{-1}. These results showed that 3 out of the 4 strains degraded over 90% of the CP. Strain AC6 demonstrated the least degradation (27.5%) and the highest increase in biomass. With these results, and the low index value (0.17) that was obtained from the degradation/biomass relationship, strain AC6 was not selected for following assays. After 72 h, the CP degradation produced by strains AC5, AC7 and AC9 was 92, 97 and 93%, respectively. The observed biomass growth of AC5, AC7 and AC9 was 37, 47 and 53% (relative to the control without CP). From these results, the index values were 2.44, 2.05 and 1.75 for strains AC5, AC7 and AC9, respectively. The CP-tolerant strains AC5 and AC7 were selected for subsequent studies.

3.2. Characterization of CP-tolerant actinobacteria

The strains selected for their tolerance to CP (50 mg L^{-1}) in agar media were identified based on 16S rDNA analysis as bacteria belonging to the phylum Actinobacteria, order Actinomycetales,

family Streptomycetaceae and genus *Streptomyces* (Table 1). A comparison of the 16S rDNA gene sequences (entire sequence compared with available sequences in GenBank) of strains AC5, AC6 and AC7 showed 99% similarity to *Streptomyces chattanoogensis*, *Streptomyces* sp. S142 and *Streptomyces olivochomogenes*, respectively. Meanwhile, strains AC9 showed 100% similarity to the sequences of the 16S rDNA genes from *Streptomyces* sp. IMCr03. To identify the phylogeny of the isolates, strains from different genera were chosen to construct the phylogenetic tree. The phylogenetic analysis (Fig. 1) based on the 16S rDNA using MEGA4 software indicates that the isolates have higher similarity with 16S rDNA gene from bacteria with ability to degrade CP, i.e., *Klebsiella* sp. *Pseudomonas putida* and the *Streptomyces radiopugmam* than with other *Streptomyces* strains reported as degraders of organochlorine pesticides.

The results of biochemical and enzymatic characterization of AC5 and AC7 *Streptomyces* CP-tolerant selected strain using Api-Coryne and Api20E showed that both Streptomycetes were positive for alkaline phosphatase, β-galactosidase, α-glucosidase, *N*-acetyl-β- glucosaminidase, catalase and citrate utilization. *Streptomyces* sp. AC5 was positive for pyrazinamidase, pyrrolidonyl arylamidase, urease, arginine dihydrolase and nitrate reduction. Unlike *Streptomyces* sp. AC5, *Streptomyces* sp. AC7 was positive for β- glucosidase activity, gelatinase and acetoin production.

Finally, the pH tolerance evaluation showed that *Streptomyces* sp. AC5 was tolerant to pH values between 5.0 and 10.0 and that *Streptomyces* sp. AC7 was tolerant to pH values between 4.0 and 10.0.

3.3. Degradation of CP in liquid medium by Streptomyces sp. strain AC5 and AC7

The results of the CP degradation, TCP production, microbial biomass, pH modification and glucose are shown in Table 2. The results showed that CP degradation by *Streptomyces* sp. AC5 and AC7 was significantly different ($p \leq 0.05$) when different concentrations of CP and different time of incubation were evaluated. In general, CP degradation after 24 h showed a disappearance of over 90%. After 72 h of incubation, concentrations of 1.70 mg L^{-1} and 2.05 mg L^{-1} of CP were detected when 25 mg L^{-1} and 50 mg L^{-1} were initially added, respectively. A similar pattern to that observed for *Streptomyces* sp. AC5 was observed for *Streptomyces* sp. AC7. The results obtained for strain AC7 showed that over 92% of CP was degraded after 24 h of incubation. In the same way, an increment of CP degradation was observed. At the end of the experiment, the concentration of CP was between 0.30 and 1.00 mg L^{-1} compared with 1.70–2.05 mg L^{-1} obtained at the same time when *Streptomyces* sp. AC5 was inoculated.

In addition to CP degradation, we evaluated the production of its major metabolite, TCP. The results showed that TCP production was significantly different ($p \leq 0.05$) at different incubation times and between the two different strains. When *Streptomyces* sp. AC5 was inoculated, TCP was detected at concentrations ranging from 0.33 mg L^{-1} at 24 h of incubation to 0.05 mg L^{-1} at 72 h and from 0.38 mg L^{-1} to 0.09 mg L^{-1} when 25 mg L^{-1} and 50 mg L^{-1} of CP were initially added, respectively. The trend showed a decrease of TCP over time, and after 96 h, no TCP residues were detected (data not shown). In contrast, when *Streptomyces* sp. AC7 was inoculated with CP, the concentration of TCP increased from 1.31 mg L^{-1} to 2.89 mg L^{-1} when 25 mg L^{-1} CP was added. Similarly, after 72 h of incubation, the concentration of TCP increased from 1.59 mg L^{-1} to 4.32 mg L^{-1} when 50 mg L^{-1} of CP was added to the liquid medium.

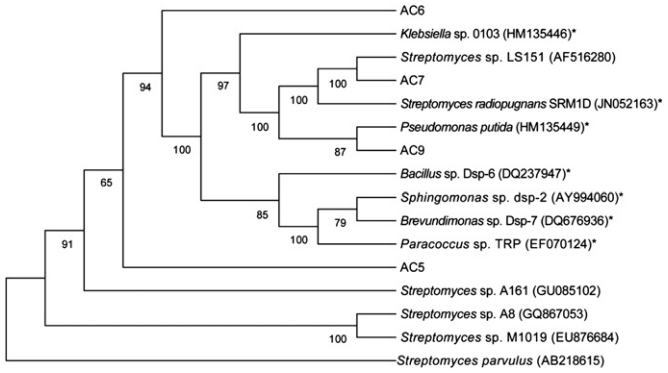

Fig. 1. Phylogenetic tree showing the affiliation of isolated chlorpyrifos-degrading actinobacteria (A5, A6, A7 and A9 strains) in relation to representative bacteria with ability to degrade organophosphorus pesticides. Asterisk indicates sequences from bacteria reported as chlorpyrifos-degraders. The neighbor-joining tree was constructed using MEGA4 and a bootstrap analysis was performed with 1000 trials.

The biomass was evaluated as the dry weight at different times of incubation. The results obtained for *Streptomyces* sp. AC5 showed different biomass concentration ($p \leq 0.05$) during incubation with CP. After 24 h of incubation, the trend from the data showed an increase in both with and without pesticide applications from an initial biomass of 1.12 mg mL^{-1} expressed as dry weight to 3.10–3.70 mg mL^{-1}. After 48 h of incubation, the biomass decreased in the treatment without CP, while in treatment with CP the biomass showed larger values than the observed at 24 h of incubation. The microbial growth for *Streptomyces* sp. AC7 was significantly different ($p \leq 0.05$). This strain doubled its biomass after 24 h of incubation from an initial biomass of 1.74 mg mL^{-1} expressed as dry weight to 2.96–3.17 mg mL^{-1} in media with and without pesticide. A larger biomass was observed after 24 h when 50 mg L^{-1} of CP was applied, and a slight decrease in biomass was observed for all treatments after 48 h, after this time a new increment was observed.

The analysis of CP degradation was accompanied by an analysis of pH changes and glucose uptake. The results showed that liquid media inoculated with *Streptomyces* sp. AC5, the pH dropped from 7.00 at time zero to 6.39 after 24 h of incubation. Then, the pH rose again. When CP was added, the pH of the media sharply dropped reaching the lowest pH value of 3.61 after 72 h of incubation. When the liquid media without and with CP were inoculated with *Streptomyces* sp. AC7, the pH values decreased from 7.00 at time zero to 6.53–6.79 after 24 h of incubation; thereafter, the pH continued to increase to a pH of 8.25 after 72 h of incubation.

The analysis of glucose uptake showed rapid substrate consumption during the first 24 h. The liquid medium initially was supplemented with 4.0 g of glucose. After 24 h, between 75 and 88% of glucose was consumed by *Streptomyces* sp. AC5, reaching nearly the complete consumption in media without CP (98%) and with CP (93%) after 72 h of incubation. A similar trend was observed for *Streptomyces* sp. AC7.

Table 1
Phylogenetic assignment of isolated strains tolerant to 50 mg L^{-1} of chlorpyrifos.

Strain	Closest relative sequence (accession number)[a]	Similarity (%)	Accession no.
AC5	*Streptomyces chattanoogensis* strain CGMCC 4.1415 (JN566019)	99	JQ289350
AC6	*Streptomyces* sp. S142 from rhizosphere (JF793513)	99	JQ289351
AC7	*Streptomyces olivochromogenes* strain xsd08162 from planted soil (FJ481078)	99	JQ289352
AC9	*Streptomyces* sp. IMCr03 from forest soil (HE577950)	100	JQ289353

[a] Based on partial sequencing of 16S rRNA gene and comparison with those present in GenBank database from National Center for Biotechnology Information (NCBI) by using BLAST.

Table 2
Residual chlorpyrifos (CP), TCP production, dry weight biomass, pH changes and residual glucose in liquid medium supplemented with CP at concentrations 0 mg L^{-1} (control), 25 mg L^{-1} and 50 mg L^{-1} inoculated with *Streptomyces* sp. strain AC5 and AC7.

Strain	Time (h)	Initial CP concentration mg L^{-1}	Residual CP* mg L^{-1} and (%)	TCP* mg L^{-1}	Dry weight biomass** mg mL^{-1}	pH**	Residual glucose** g L^{-1}
AC5	0		24.5 ± 0.50 (98%)^{a}	0.01 ± 0.00^{d}	1.12 ± 0.10^{c}	7.00 ± 0.01^{b}	4.00 ± 0.01^{a}
	24	0	–	–	3.70 ± 0.16^{a}	6.39 ± 0.09^{ab}	0.49 ± 0.09^{c}
		25	2.55 ± 0.65; (10.2%)^{bcde}	0.32 ± 0.06^{cd}	3.20 ± 0.16^{a}	3.83 ± 0.08^{c}	0.99 ± 0.03^{b}
		50	3.90 ± 0.30; (7.8%)b	0.38 ± 0.00^{cd}	3.10 ± 0.02^{ab}	3.70 ± 0.04^{c}	1.01 ± 0.05^{b}
	48	0	–	–	2.68 ± 0.37^{b}	7.71 ± 0.05^{a}	0.23 ± 0.03^{c}
		25	1.80 ± 0.20; (7.2%)^{bcde}	0.46 ± 0.06^{cd}	3.74 ± 0.17^{a}	3.96 ± 0.04^{c}	0.40 ± 0.04^{c}
		50	2.90 ± 0.80; (5.8%)^{bcd}	0.28 ± 0.02^{cd}	3.77 ± 0.03^{a}	3.89 ± 0.39^{c}	0.43 ± 0.21^{c}
	72	0	–	–	2.96 ± 0.05^{b}	8.00 ± 0.21^{a}	0.08 ± 0.01^{d}
		25	1.70 ± 0.20; (6.8%)^{bcde}	0.05 ± 0.00^{d}	3.88 ± 0.33^{a}	3.86 ± 0.17^{c}	0.34 ± 0.07^{c}
		50	2.05 ± 0.10; (4.1%)^{bcde}	0.09 ± 0.01^{d}	3.67 ± 0.37^{a}	3.61 ± 0.02^{c}	0.27 ± 0.17^{c}
AC7	0		24.5 ± 0.50 (98%)^{a}	0.01 ± 0.01^{d}	1.76 ± 0.15^{c}	7.00 ± 0.20^{abc}	4.00 ± 0.50^{a}
	24	0	–	–	2.96 ± 0.10^{ab}	6.60 ± 0.02^{c}	0.93 ± 0.52^{b}
		25	2.00 ± 0.01; (8.0%)^{bcde}	1.31 ± 0.14^{bcd}	2.42 ± 0.20^{b}	6.79 ± 0.14^{c}	1.20 ± 0.66^{b}
		50	3.60 ± 0.20; (7.2%)^{bc}	1.59 ± 0.01^{bcd}	3.17 ± 0.17^{a}	6.53 ± 0.18^{c}	0.56 ± 0.05^{bc}
	48	0	–	–	1.28 ± 0.01^{d}	7.10 ± 0.04^{abc}	0.24 ± 0.02^{d}
		25	1.40 ± 0.05; (5.6%)^{cde}	1.87 ± 0.36^{bc}	1.65 ± 0.09^{c}	7.01 ± 0.03^{abc}	0.76 ± 0.17^{bc}
		50	1.80 ± 0.25; (3.6%)^{bcde}	2.43 ± 0.86^{b}	2.18 ± 0.21^{b}	6.94 ± 0.01^{bc}	0.18 ± 0.03^{e}
	72	0	–	–	2.10 ± 0.18^{b}	8.12 ± 0.51^{ab}	0.14 ± 0.01^{e}
		25	0.30 ± 0.05; (1.2%)^{e}	2.89 ± 0.14^{ab}	2.09 ± 0.01^{b}	8.25 ± 0.33^{a}	0.15 ± 0.01^{e}
		50	1.00 ± 0.10; (2.0%)^{de}	4.32 ± 0.67^{a}	2.34 ± 0.03^{b}	8.11 ± 0.20^{ab}	0.16 ± 0.01^{e}

The average values and the standard error are presented ($n = 3$); (–) = not determined.
Different letters indicate significantly different values, ANOVA with Tukey test ($p \leq 0.05$).
*The analyses were done in the same column between strains.
**The analyses were done in the same column for each strain separately.

The results obtained through CP extraction from cells showed an adsorption of 12.5% and 7.3% after 24 h of incubation when 25 mg L^{-1} and 50 mg L^{-1} CP was applied, respectively. There were no significant differences ($p \leq 0.05$) between strains.

Finally, the analysis of organic acids in the supernatant of liquid medium inoculated with CP and *Streptomyces* sp. strain AC5 and AC7 showed that CP exposition after 24 h of incubation caused an excretion of organic acids mixture constituted of oxalic acid, malic acid and pyruvate acid. In both strains the same mixture of organic acids was observed but in different concentrations. For *Streptomyces* sp. AC7 a concentration of 5.16 mg L^{-1}, 6.83 mg L^{-1} and 2.20 mg L^{-1} for oxalic, malic and pyruvate, respectively. While, for *Streptomyces* sp. AC5 a concentration of 8.69 mg L^{-1}, 26.81 mg L^{-1} and 324.64 mg L^{-1} of oxalic, malic and pyruvate, respectively, was measured.

4. Discussion

Actinobacteria are widely distributed in natural and man-made environments (Goodfellow and Williams, 1983). In this study, agricultural soil with a history of CP applications was used to isolate actinobacteria with potential for the degradation of CP. Nearly 80% of the isolated bacteria showed some degree of CP tolerance. However, four strains that were tolerant to 50 mg L^{-1} of CP were selected and identified as belonging to *Streptomyces* genus, which are dominant actinobacteria in soil, representing up to 20% of soil bacteria (Seong et al., 2001; Kothe et al., 2005).

Somewhat surprising, the phylogenetic analysis of isolates showed a closer relation with CP-degrading bacteria but other genus of bacteria, i.e., *Klebsiella* sp. and *P. putida*, compared with *Streptomyces* strains with ability to degrade organochlorine pesticides (Fig. 1). We may speculate that the studied *Streptomyces* are close to other bacteria independent from their phylogenetic affiliation. However, further methods including ERIC- PCR technique, physiological and biochemical characterization, degrading characters and *mpd* gene sequence comparison could be resolved to a much higher degree as compared to single approaches (Li et al., 2008).

The results obtained from the quantitative screening demonstrated that all of the assayed actinobacteria (strains AC5, AC6, AC7 and AC9) were able to grow and to remove CP. In the same way observed by Benimeli et al. (2007) and Fuentes et al. (2011), no linear relationship was found between pesticide degradation and microbial growth, so it was necessary to examine the relationship between both of these parameters to select the best strains for future assays. For our case, the actinobacteria with the highest ratio were chosen, corresponding to a high degradation of CP and low biomass production. As a result, *Streptomyces* sp. AC5 and AC7 were selected due to their efficiency at degrading CP with a minor amount of biomass. Microorganisms that are capable of pollutant biodegradation are typically already present in contaminated soils (Fantroussi and Agathos, 2005). Therefore, the use of these actinobacteria for the bioremediation of matrices contaminated by pesticides is an attractive approach because these microorganisms have already adapted to the habitat. The rapid CP degradation by these strains that were isolated from agricultural soil, which had received treatment with CP, could indicate the ability of the organisms to adapt to the environment conditions. Repeated applications of pesticides in agricultural soils, specifically OPs, lead to the enhanced biodegradation of the compounds (Singh, 2008).

Actinobacteria such as *Streptomyces* sp. are metabolically versatile bacteria that play an important role in the biodegradation of organic compounds (Castillo et al., 2006; Fuentes et al., 2011). Some strains of *Streptomyces* have been reported to degrade chloroacetanilide (Sette et al., 2004, 2005) pyrethroid (Lin et al., 2011) and organochlorine pesticides (Fuentes et al., 2010, 2011). In this study, the evaluation of CP degradation at concentrations of 25 mg L^{-1} and 50 mg L^{-1} after 24, 48 and 72 h by *Streptomyces* sp. AC5 and AC7 showed a high and fast degradation of CP during the first 24 h of incubation. According to our results, removal of CP from the liquid medium would be mainly caused by degradation process and not by the adsorption process in the cells. In the biomass between 9 and 12% of CP was adsorbed. Similarly at these results, about 9.04% of CP extracted from the biomass of a cyanobacterium was reported by Singh et al. (2011).

The similar pattern of CP degradation showed by studied strains characterized both to be positive for alkaline phosphatase could explain this result due this enzyme is a phosphomonoesterase

attributed to act in the CP degradation pathway hydrolyzing O–P bonds leaving phosphorus atom available for uptake as a source of phosphorus and to release ethanol for utilization as a carbon source (Singh et al., 2004; Singh and Walker, 2006). Such short times for the complete degradation of CP as those that were obtained in this study have also been observed elsewhere. For example, Xu et al. (2007) showed that 100 mg L^{-1} of CP required only 24 h for the complete mineralization by *Serratia* sp. and *Trichosporon* sp. Yang et al. (2005) demonstrated the rapid degradation of 100 mg L^{-1} CP during the first two days of incubation with a degradation rate of 20 mg CP L^{-1} d^{-1} by a bacterium that was isolated from contaminated soil. This result was also accompanied by microbial growth, similar to that observed for *Streptomyces* sp. AC5 and AC7. Likewise, Xu et al. (2008) observed the complete mineralization of 50 mg L^{-1} of CP within 4 d in addition to the accumulation of TCP and bacterial growth. Moreover, in the same study, 50 mg L^{-1} of CP was rapidly degraded in the first 3 h by cell-free extracts that were pre-cultured with CP. Recently, Sasikala et al. (2012) reported the degradation of 500 mg L^{-1} CP by a bacterial consortium that consisted of *Klebsiella* sp., *Pseudomona stutzeri* and *P. putida* that had been isolated from contaminated agricultural soil. In the same study, an actinobacteria identified as *Streptomyces radiopugnans* was capable of degrading 25% of added CP. As was mentioned before, diverse bacteria present the ability to degrade CP at high concentrations. However, our results constitute a new report of CP degradation by CP-degrading isolates from the genus *Streptomyces*.

Previous researchers reported that the removal of CP occurred with the formation of metabolites such as CP-oxon, 3,5,6-trichloro-2-methoxypyridine, 2-chloro-6-hydroxypyridine and TCP, this latter characterized as the main degradation product that is accumulated in pure cultures, water and soils (Singh and Walker, 2006; Xu et al., 2008; Li et al., 2010). In this study, a different trend of TCP production by *Streptomyces* sp. AC5 and AC7 was observed. This could indicate that CP degradation in these strains could follow different metabolic pathways, as *Streptomyces* sp. AC5 and AC7 appeared to be different species based on 16S rDNA (Table 1) and biochemical profiles.

The mechanism of TCP degradation in bacteria is fairly understood and a number of degradation products such as chlorodihydro-2-pyridone, dihydroxypyridine, tetrahydro-2- pyridone, maleamide semialdehyde, maleamic acid and pyruvic acid have been identified (Singh and Walker, 2006). Recent studies investigating the bacterial capacity for TCP degradation have been conducted. For example, Li et al. (2010) recently showed that *Ralstonia* sp. strain T6 has the ability to completely eliminate 100 mg L^{-1} TCP within 12 h. Though, a decrease in the biomass during the first 4 h was observed because of the inhibition effect of TCP, most likely due to the antimicrobial effect of the compound. However, the biomass gradually increased as the strain acclimated to the inhibition of TCP (Li et al., 2010). According with our results, the elimination of TCP during that time could indicate that *Streptomyces* sp. AC5 possesses the ability to degrade TCP. This could be corroborated by the presence of high concentration of pyruvate (324.64 mg L^{-1}) in the medium after 24 h of incubation. According to Singh and Walker (2006) the CP degradation through TCP pathway finished with pyruvic acid as a metabolite prior to start the Krebs cycle with consequent release of CO_2 and water. In contrast, the TCP accumulation with AC7 during the incubation time could indicate the inefficacy of this bacterium to degrade this compound. In this case a concentration of 2.20 mg L^{-1} of pyruvate was founded. Little literature is available on the microbial metabolism of TCP in liquid medium (Singh and Walker, 2006). Recently, the mineralization of TCP has been reported for *Paracoccus* sp. strain TRP (Xu et al., 2008), *Alcaligenes faecalis* (Yang et al., 2005) and *Burkholderia* sp. strain KR100 (Kim and Ahn, 2009) that were isolated from soils. It is postulated that TCP is metabolized by a reductive dechlorination pathway (Feng et al., 1998), where degrading enzymes might be inducible (Xu et al., 2008). TCP degradation by actinobacteria has not yet been studied. Characterizing the pathways of degradation and identifying the enzymes that are involved in the process are further matters to investigate to clarify the ability of the studied actinobacteria to degrade CP and TCP.

Analyses of pH and glucose uptake in the liquid medium were conducted to obtain a better understanding of CP degradation by *Streptomyces* sp. strains AC5 and AC7. The decrease in pH values after 24 h in the liquid media without CP, inoculated with strains AC5 and AC7 coincided with the time at which about complete glucose uptake was observed. This is a very common result that is caused by the addition of easily biodegradable substrates. As a consequence of microbial glucose metabolism, a decrease in the pH of the medium is observed due to the accumulation of organic acids (Kontro et al., 2005; Cycón et al., 2009). The excretion of significant amounts of organic acids metabolites into the extracellular medium has been observed in cultures of different *Streptomyces* sp. (Madden et al., 1996), and this excretion in microorganism occurs specially in situations where nutrients may be limiting (Jones, 1998). In this study, we identified the production or excretion by *Streptomyces* sp. AC5 and AC7 of three organic acids, these were: oxalic acid, malic acid and pyruvate acid, which could be re-assimilated by the microorganisms after the exhaustion of glucose (Madden et al., 1996; Corvini et al., 2004). An increase of biomass was observed for *Streptomyces* sp. AC7 after 72 h and the pH rose to values over 8.0. When *Streptomyces* sp. AC5 was inoculated with CP a strong decrease of pH values was observed for all incubation time. Variations in pH of liquid media that has been contaminated with CP at concentrations between 10 and 50 mg L^{-1} and inoculated with *Pseudomanas aeroginosa* were previously observed by Fulekar and Geetha (2008). According to our results, the high concentration of organic acids excreted by *Streptomyces* sp. AC5 favored the liquid medium acidification. There was no increase in biomass at the different times when the strain was exposed to CP; therefore, we may exclude re-assimilation of organic acids from the medium.

CP is co-metabolically hydrolyzed to TCP in liquid media by bacteria that need extra carbon sources (Singh and Walker, 2006; Xu et al., 2008). In this study, nearly the complete degradation of CP was observed after the first 24 h of incubation, and >90% glucose was removed from the media. This indicated that glucose and CP were simultaneously consumed and that CP could be degraded co-metabolically by strain AC5 and AC7, which might signify the environmental adaptation of these bacteria. A more rapid degradation of CP in the presence of an additional carbon source was demonstrated by *Paraccocus* sp. strain TRP (Xu et al., 2008). Co-metabolism in the presence of a carbon source occurs in actinobacteria, and it has been shown that most pesticides can serve as carbon, nitrogen, and/or phosphorous sources via partial transformation reactions. Based on many studies in different microorganisms, co-metabolism may be the most widespread mechanism for pesticide degradation (De Schrijver and De Mot, 1999).

5. Conclusion

In conclusion, we describe here two bacterial isolates from soil that had received continuous applications of CP that were capable of degrading the compound. Based on 16S rDNA gene, they were identified as actinobacteria of genus *Streptomyces* sp. To our knowledge, this is one more of the few reports that have isolated native CP-degrading bacteria from this genus. The two species of *Streptomyces* sp. strains AC5 and AC7 were able to rapidly degrade

CP from the culture medium, which was confirmed with the appearance of TCP. Moreover, the genetic and biochemical analysis, the changes of pH in culture medium associated to organic acids production and differences in the production of TCP suggest that the isolates are functionally different species of *Streptomyces*. The efficient bioremediation of contaminated soils and water could likely be obtained with the use of these *Streptomyces* sp. However, future studies are necessary to further the knowledge about these bacteria and the metabolism that is involved in the degradation pathways of CP and their metabolite TCP.

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