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ORIGINAL PAPER

Identification and characterization of chlorpyrifos-methyl and 3,5,6-trichloro-2-pyridinol degrading *Burkholderia* sp. strain KR100

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Abstract A chlorpyrifos-methyl (CM) degrading bacterium (designated strain KR100) was isolated from a Korean rice paddy soil and was further tested for its sensitivity against eight commercial antibiotics. Based on morphological, biochemical, and molecular characteristics, this bacterium showed greatest similarity to members of the order Burkholderiales and was shown to be most closely related to members of the *Burkholderia cepacia* group. Strain KR100 hydrolyzed CM to 3,5,6-trichloro-2-pyridinol (TCP) and utilized TCP as the sole source of carbon for its growth. The isolate was also able to degrade chlorpyrifos, dimethoate, fenitrothion, malathion, and monocrotophos at 300 μg/ml but diazinon, dicrotophos, parathion, and parathion-methyl at 100 μg/ml. The ability to degrade CM was found to be encoded on a single plasmid of ~50 kb, pKR1. Genes encoding resistance to amphotericin B, polymixin B sulfate, and tetracycline were also located on the plasmid. This bacterium merits further study as a potential biological agent for the remediation of soil, water, or crop contaminated with organophosphorus compounds because of its greater biodegradation activity and its broad specificity against a range of organophosphorus insecticides.

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Abbreviations

CM	Chlorpyrifos-methyl
TCP	3,5,6-Trichloro-2-pyridinol
OP	Organophosphorus insecticide
MSM	Mineral salts basal medium
PTYG	Peptone–tryptone–yeast extract–glucose

Introduction

Chlorpyrifos-methyl (CM), *O*,*O*-dimethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate, is a nonsystemic, broad-spectrum organophosphorus insecticide (OP) with contact, stomach, and respiratory action (Tomlin 2003). CM has been one of the insecticides commonly used worldwide since 1985. In Korea, ~62 tons are used annually in agriculture (KCPA 2005). Contamination of soil by CM can result from bulk handling in the farmyard and rinsing of containers and accidental release may occasionally lead to the contamination of surface water and groundwater. Additionally, the excessive and frequent application of CM may result in high levels of CM residues accumulated on agricultural crops, which poses a potential health hazard to consumers. Maximum residue limits of CM have been

recommended by the joint FAO/WHO meeting on pesticide residues (IPCS 2008). These problems highlight the need for bioremediation agents for removal of CM and residues from the contaminated soil, water system, or crop.

Microorganisms capable of degrading OPs are a potential for bioremediation because they are considered to play a significant role in breaking down and detoxifying them in the environment (Laveglia and Dahm 1977; Chapalamadugu and Chaudhry 1992; Singh and Walker 2006). Because of this, much effort has been focused on them as potential sources of biological agents for the remediation of soil, water, or crop contaminated with OPs. The hydrolytic degradation of CM has been characterized in the aquatic and soil environments (Meikle and Youngson 1978; Tomlin 2003). The most common pathway of hydrolytic degradation of the phosphorothioate involves formation of 3,5,6-trichloro-2-pyridinol (TCP) by microorganisms. Particularly, TCP is a charged molecule at neutral pH and more leachable into groundwater and surface water than the parent compound (Manclús and Montoya 1995). It is moderately mobile due to its greater water solubility, which can cause the widespread contamination in soil and aquatic environment (Yang et al. 2005). Unlike other OPs, there are reports of the resistance of chlorpyrifos to enhanced degradation in soil (Racke et al. 1990; Singh et al. 2004). It was suggested that the accumulation of TCP, which has antimicrobial activity, acts as a buffer in the soil and prevents the proliferation of chlorpyrifos degrading microorganisms (Racke et al. 1990). There have been no studies on microbial degradation of both CM and TCP, although degradation of TCP by *Alcaligenes faecalis* has been reported previously (Yang et al. 2005). Additionally, antibiotics have been reported in foodstuffs through various routes, which is of considerable significance for public health (Khan and Malik 2001).

The present study was aimed at isolating and characterizing a *Burkholderia* sp. strain KR100 capable of degrading CM and TCP as a sole source of carbon from the rice paddy soil and at investigating its ability to degrade other OP and two carbamate insecticides. We demonstrated that the gene for the CM degradation is encoded on a single plasmid in the bacterium. Also, the sensitivity of the bacteria to the eight commercial antibiotics is discussed.

Materials and methods

Chemicals

Twelve insecticides used in this study were as follows: CM (99.3% purity), carbaryl (99%), chlorpyrifos (99.5%), diazinon (99.5%), dicrotophos (98%), dimethoate (99%), fenitrothion (95%), malathion (99.0%), monocrotophos (99.5%), and parathion (99%) purchased from Chem Service (West Chester, PA); parathion-methyl (99.9%) purchased from Supelco (Bellefonte, PA); and furathiocarb (97.9%) purchased from Riedel-de Haen (Seelze, Germany). TCP (99.0%) was obtained from Chem Service. Eight antibiotics used in this study were as follows: amoxicillin, amphotericin B, metronidazole, polymixin B sulfate, tetracycline, trimethoprim, and vancomycine supplied by Sigma–Aldrich (St Louis, MO); and chloramphenicol supplied by Fluka (Buchs, Switzerland). All other chemicals and reagents were of analytical grade and available commercially.

Isolation of a CM degrading bacterium by enrichment culture

Mineral salts basal medium (MSM) (Stanier et al. 1966) was used in both enrichment culture of agricultural soils and liquid culture of isolated bacteria. Peptone–tryptone–yeast extract–glucose (PTYG) agar medium (Yi et al. 2000) was used for bacterial purification and colony production for the PCR.

Soil samples were taken from upland fields (161 sites), orchards (44 sites), and rice paddy fields (68 sites) in Korea. Samples were taken from the top 15 cm of soil and kept at 4°C until use. A 10-g soil sample from each site was homogenized in 10 ml of 0.85% saline solution by shaking the preparation on a rotary shaker at 25°C and 100 rpm. A tenfold dilution series was prepared and 0.1 ml of each dilution was inoculated into test tubes containing 3 ml of MSM supplemented with 300 μg/ml of CM as the sole carbon source. The tubes were incubated on a rotary shaker at 29°C for 2 weeks at 100 rpm. The culture of the terminal positive tube showing the growth was enriched by two serial transfers into fresh media. Each enriched culture was streaked onto PTYG agar plates, and single colonies were then tested for CM utilization as a sole source of carbon in fresh MSM

with 300 μg/ml of CM before strain purification. Following this exercise, one efficient CM degrading organism from rice paddy soil, designated strain KR100, was selected for further studies.

Bacterial growth and degradation of CM and TCP

Strain KR100 was precultured in Erlenmeyer flasks (500 ml) containing 200 ml of MSM with 300 μg/ml of CM and 300 and 100 μg/ml of TCP. Inoculated flasks were incubated 1 week at 29°C on a rotary shaker at 100 rpm. Samples of cultures were periodically withdrawn and analyzed. Bacterial density was determined by measuring the optical density at 550 nm (OD_{550}). Noninoculated medium with CP and TCP was used as a control. Degradation of CM and TCP was analyzed spectrophotometrically at 300 and 320 nm, respectively. All experiments were replicated three times.

Bacterial identification

Strain KR100 was further identified and characterized by using standard biochemical methods (George et al. 2005) combined with 16S rRNA gene sequence analysis. Chromosomal DNA was isolated from bacterial cells grown on PTYG agar plates overnight as described previously (Mallick et al. 1999). The guanine plus cytosine (G + C) content of bacterial DNA was determined according to the method of Tamaoka and Komagata (1984).

The 16S rRNA gene of strain KR100 was PCR amplified using the 27f and 1492r universal primers (Lane 1991). Sequencing of PCR fragments was performed by using the 27f, 1492r, and 519r universal primers (Lane 1991) after purification of the PCR products with a QIAquick PCR purification kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. The sequence of the 16S rRNA (~500 bp) of the strain was compared with other bacterial 16S rRNA gene sequences in the GenBank database using the NCBI Blast program. Multiple alignments of sequences, construction of a neighbor-joining phylogenetic tree, and a bootstrap analysis for evaluation of the phylogenetic topology were accomplished using Clustal X program (ftp://ftp.ebi.ac.uk/pub/software/unix/clustalx/) and MEGA 3.1 software (www.megasoftware.net).

Axenic culture experiment

After growth in MSM with or without CM to produce cells with their CM metabolism induced or uninduced, respectively, cells were harvested, washed twice with an equal volume of 15 mM phosphate buffer (pH 7.0), and resuspended in the same buffer (Yi et al. 2000). Aliquots of the suspended cells were inoculated into flasks containing 200 ml of MSM with 300 μg/ml of CM at a final density at 550 nm of about 0.005. All cultures were incubated at 29°C on a rotary shaker at 100 rpm. Aliquots of cultures were periodically withdrawn to determine cell growth and degradation of CM as stated above.

Substrate range

Cross-feeding studies with other insecticides were also performed by using the intact cell and cell extract. The insecticides used in this study were nine OPs (chlorpyrifos, diazinon, dicrotophos, dimethoate, fenitrothion, malathion, monocrotophos, parathion, and parathion-methyl) and two carbamates (carbaryl and furathiocarb). For intact cell, the liquid medium was supplemented with the test insecticide at 100 or 300 μg/ml and incubated 1 week at 29°C on a rotary shaker at 100 rpm. For cell extract, cells were grown in PTYG for 24 h and then were harvested by centrifugation (14,000 rpm, 10 min, 4°C). The cells were washed twice with 50 mM potassium phosphate buffer (pH 7.0). The cell paste was resuspended in the same buffer and then disrupted by a bead beater 1 h at 4°C. Cell debris was removed by centrifugation as stated above. The cell extract was assayed in a reaction mixture containing 100 μg/ml insecticides tested (Nine organophosphates and two carbamates), 50 mM potassium phosphate buffer (pH 7.0), and the cell extract in a total volume of 1.0 ml. The reaction was started by the addition of cell extract and carried out at 29°C at 30 min. Hydrolysis of these insecticides was measured spectrophotometrically.

Plasmid curing and detection

For plasmid curing, single colonies of the strain KR100, grown on MSM with 300 μg/ml CM as a sole carbon source, were inoculated into PTYG broth containing mitomycin C (0.6 μg/ml) and shaken at

29°C overnight (Hayatsu et al. 2000). Dilutions were made from the overnight culture and plated out onto PTYG agar. After 1 day of incubation at 29°C, a mutant of KR100 unable to degrade CM was selected.

For plasmid detection, plasmids from strain KR100 and its derivative mutant were isolated by using a pLASmix-minipreps kit (Amersham, Uppsala, Sweden) according to the manufacturer's instructions. Electrophoresis in 1% agarose was carried out at 40 V for 4 h, and plasmid DNA was visualized by ethidium bromide staining.

Antibiotic sensitivity assay

Strain KR100 and its cured strain KR101 were also tested for their sensitivity to the eight antibiotics by using a paper disk diffusion method (Yeon et al. 2007). Briefly, 0.1 ml cultures of the test organisms were seeded on PTYG agar plates. A sample (30, 10, and 6 μg) in methanol (0.1 ml) was applied by a micropipet to an Advantec paper disk (8 mm in diameter and 1 mm in thickness; Toyo Roshi, Japan). After they were dried in a fume hood, the disks were placed on the agar surface inoculated with the test organism. All plates were incubated at 37°C for 24 h. Control disks received methanol (0.1 ml). Diameters of inhibition zones were recorded. All tests of growth inhibition were replicated three times.

The inhibitory responses were classified as follows: susceptible, zone diameter $\geq$20 mm; intermediate, zone diameter 13–19 mm; resistant, zone diameter 9–12 mm; and no inhibition.

Metabolite analysis

A Shimadzu GC-2010 gas chromatograph-GCMS QP2010 mass spectrometer (Osaka, Japan) were used to identify the degradation products of CM in strain KR100. Cultures containing 300 μg/ml of CM were extracted with equal volumes of ethyl acetate. The organic phase was dehydrated, by passing it through anhydrous Na_2SO_4, and concentrated to dryness by rotary evaporation at 40°C. Appropriate dilutions of the sample extract were then analyzed with a gas chromatograph equipped with a FID and a 30 m × 0.32 mm i.d. (d_f = 0.25 μm) DB-1 MS capillary column (J&W Scientific, Folsom, CA).

Scanning electron microscopy

Strain KR100 was fixed with same amount of 2.5% glutaldehyde (v/v) that diluted 2.5% paraformaldehyde (w/v) and 0.1 M PBS buffer (pH 7.2) at 4°C for 4 h, and then washed three times with same buffer for 30 min. Second fixing was performed with 1% OsO_4 (w/v) in 0.1 M PBS buffer (pH 7.2) in room temperature for 1 h. Fixed samples were washed several times with same buffer, and samples were dehydration in each ethanol solution. Finally, samples were substituted to isoamyl acetate and dried in E3000 critical point drying machine (Bio–Rad, Cambridge, MA). Specimen was photographed using a SEM 515 scanning electron microscope (Philips, Amsterdam, The Netherlands) at 25 kV.

Results

Strain isolation and identification

Strain KR100 isolated through enrichment procedure from the Korean rice paddy soil was capable of utilizing CM as a sole source of carbon. This strain was a nonmotile, Gram negative, and nonspore-forming bacterium with a small straight rod shape (0.3–0.5 μm in length and 0.2–0.3 μm in width). Biochemically, the strain was catalase positive, oxidase negative, and urease positive. It metabolized glucose oxidatively and did not reduce nitrate. This organism was able to utilize for growth the following compounds as the carbon source: fructose, glucose, glycerol, mannitol, mannose, melibiose, raffinose, sorbitol, and xylose. It was not able to grow on arabinose, erythrose, sorbose, and Simmon's citrate. The DNA G + C content of strain KR100 was 62.3 ± 0.2 mol %. The sequence of ~500 bp of the 16S rRNA gene from strain KR100 showed greatest similarity to the sequences of the 16S rRNA genes from members of the order Burkholderiales. The results indicated that 99% similarity was seen to *Burkholderia cepacia* (GenBank accession no. DQ812969) and *B. cepacia* (GenBank accession no. NC008392). The phylogenetic analysis (Fig. 1) based on the 16S rRNA analysis using MEGA 3.1 again indicate that the isolate is most similar to *B. cepacia* group. Based on these data, the isolate was designated *Burkholderia* sp. strain KR100.

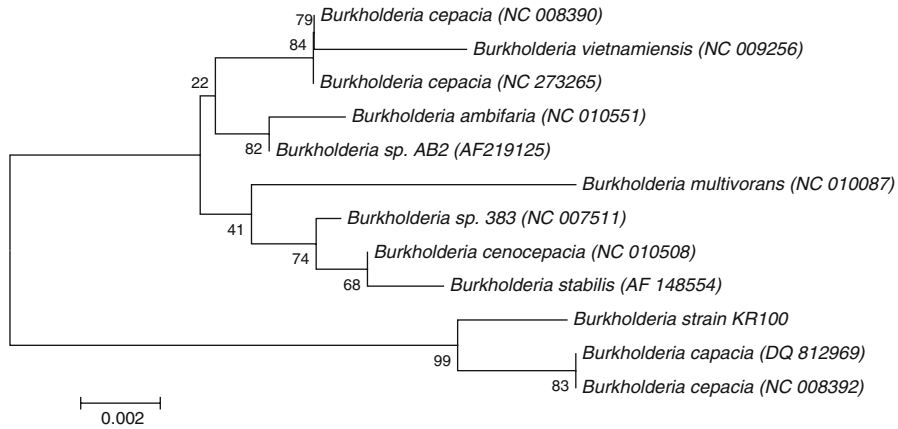

Fig. 1 Phylogenetic tree based on the 16S rRNA gene sequences of chlorpyrifos-methyl degrading strain KR100. GenBank accession numbers are given in *parentheses*. The *scale bar* indicates 0.002 substitutions per nucleotide position

Bacterial growth and biodegradation of CM and TCP

Figure 2 shows the time course for the bacterial growth and degradation of CM by *Burkholderia* sp. strain KR100 in MSM with 300 μg/ml of CM. Colorimetric analysis and optical density measurements of the enrichment culture confirmed substantial disappearance of CM with a simultaneous increase in bacterial mass. CM was almost completely degraded within 132 h. Two metabolites detected in the culture extracts during 132 h of incubation were identified as a major metabolite TCP and a minor metabolite dimethylthiophosphoric acid on the basis of structural confirmation by GC and GC–MS (data not shown).

Because of TCP formation as above, typical growth and degradation of TCP by *Burkholderia* sp. strain KR100 in MSM with 300 and 100 μg/ml of TCP were examined (Fig. 3). Bacterial growth was accompanied by the degradation of TCP at 100 μg/ml, and TCP almost completely disappeared after 144 h of cultivation. However, the strain did not degrade TCP at 300 μg/ml.

To understand axenic growth patterns of *Burkholderia* sp. strain KR100, the strain was inoculated into MSM containing 300 μg/ml CM under CM-induced and -uninduced conditions (Fig. 4). Under uninduced condition, strain KR100 showed lag period of ~84 h and thereafter began to grow exponentially. When adapted to CM metabolism, the strain

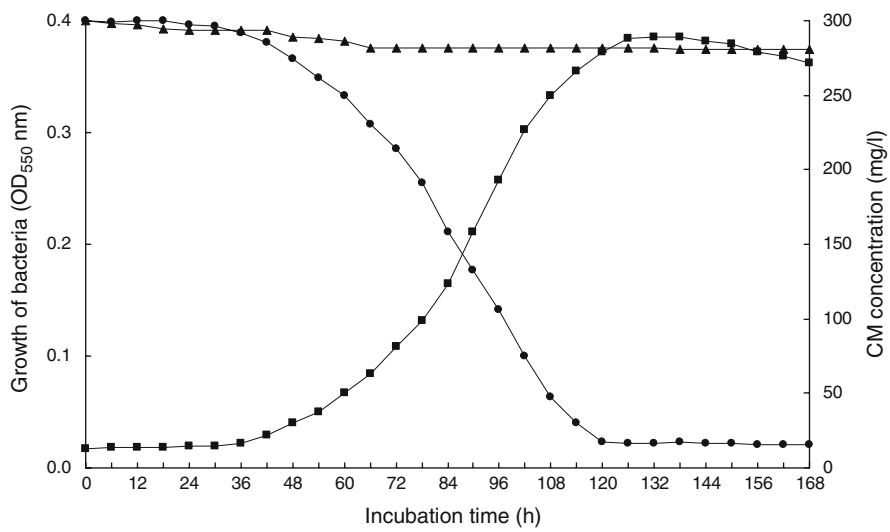

Fig. 2 Bacterial growth and corresponding degradation of chlorpyrifos-methyl (CM) as a sole carbon source for *Burkholderia* sp. strain KR100. ■ The optical density (OD) of culture; ● CM concentration in the medium; ▲ CM concentration in an noninoculated control

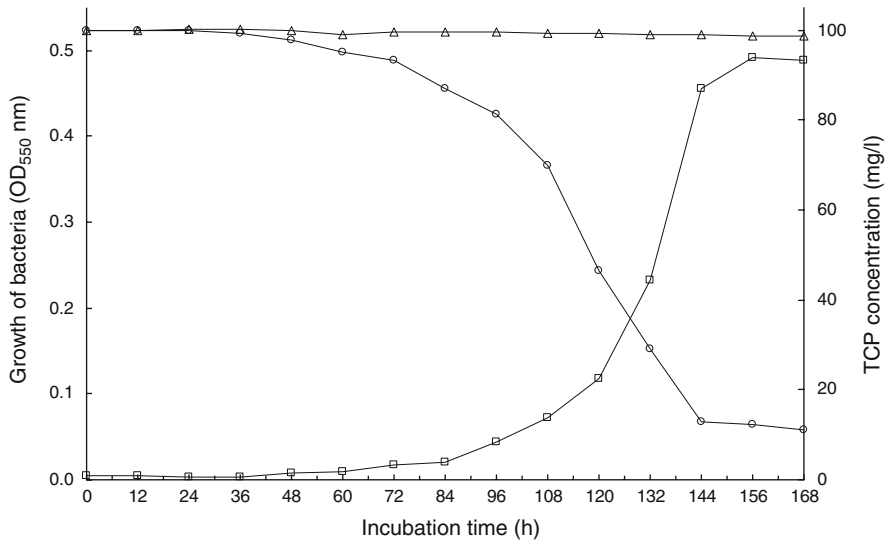

Fig. 3 Bacterial growth and corresponding degradation of 3,5,6-trichloro-2-pyridinol (TCP) as a sole carbon source for *Burkholderia* sp. strain KR100. □ The optical density (OD) of culture; ○ TCP concentration in the medium; △ TCP concentration in an noninoculated control

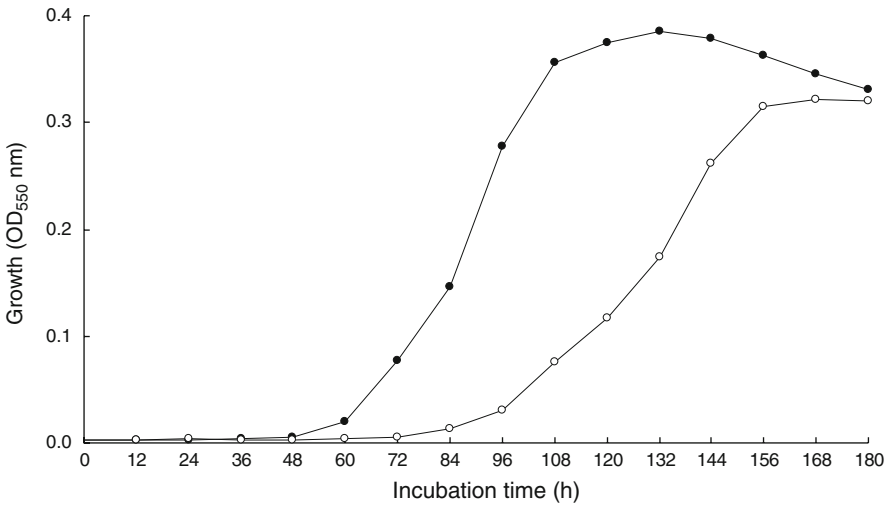

Fig. 4 Growth patterns of *Burkholderia* sp. strain KR100 in axenic cultures. The inoculated bacteria were either adapted (*solid circle*) or unadapted (*open circle*) to chlorpyrifos-methyl

exhibited significantly reduced lag phase and grew fast on MSM with CM.

Substrate specificity

The degradation patterns of different insecticides in MSM cultures inoculated with *Burkholderia* sp. strain KR100 were examined, which was determined by measuring the decrease of substrate concentration. Strain KR100 was generally restricted in its substrate utilization abilities (Table 1). It was able to degrade the OPs having dimethyl phosphorothionate side chain (fenitrothion), diethyl phosphorothionate side chain (chlorpyrifos), dimethyl phosphorodithionate side chains (dimethoate and malathion), and dimethyl phosphate side chain (monocrotophos) at 300 μg/ml. The strain degraded dimethyl phosphorothioate parathion-methyl, diethyl phosphorothioate diazinon and parathion, and dimethyl phosphate dicrotophos at 100 μg/ml but not at 300 μg/ml. Two carbamate insecticides (carbaryl and furathiocarb) were almost not degraded by strain KR100.

The degradation of different insecticides in MSM cultures was examined by using *Burkholderia* sp. strain KR100 cell extract (Table 1). At 100 μg/ml, the cell extract was able to degrade >70% of chlorpyrifos, dicrotophpos, dimethoate, fenitrothion, malathion and monocrotophos. It degraded approximately 60% of

Table 1 Degradation of different organophosphorus and carbamate insecticides by *Burkholderia* sp. strain KR100 intact cell and cell extract

Substrate	Concentration (μg/ml)	Extent of degradation (%)	
		Intact cell	Cell extract
Chlorpyrifos	300	89.8	–
	100	–[a]	77.6
Chlorpyrifos-methyl	100	97.1	63.3
Diazinon	300	5.4	–
	100	90.0	50.3
Dicrotophos	300	3.0	–
	100	87.8	71.1
Dimethoate	300	94.4	–
	100	–	81.8
Fenitrothion	300	83.7	–
	100	–	74.6
Malathion	300	82.3	–
	100	–	78.6
Monocrotophos	300	96.4	–
	100	–	78.9
Parathion	300	4.1	–
	100	90.0	52.5
Parathion-methyl	300	5.0	–
	100	84.9	62.9
Carbaryl	300	9.7	–
	100	–	30.2
Furathiocarb	300	8.2	–
	100	–	19.3

[a] Not determined

CM and parathion-methyl but approximately 50% of diazinon and parathion. Carbaryl and furathiocarb treatment resulted in 30 and 19% degradation, respectively, indicating that these carbamate insecticides might be toxic to the cell metabolism.

Plasmid curing

Burkholderia sp. strain KR100 was examined for its plasmid content by the procedure described in "Materials and Methods" section because the involvement of catabolic plasmid(s) in the degradation of a number of organic compounds has been noted (Sayler et al. 1990; Singh and Walker 2006). A plasmid of ~50 kb in size was found to be present and was designated pKR1 (Fig. 5, lane 2). A curing

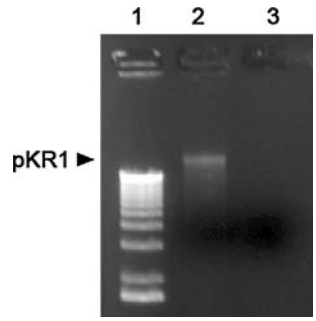

Fig. 5 Agarose gel electrophoresis of a plasmid isolated from strain KR100 and its plasmid-cured derivative. *Lanes 1*, molecular mass ladder; *2*, strain KR100; *3*, strain KR101

experiment with mitomycin C was used to determine whether the CM metabolism is controlled by pKR1 present in strain KR100. The cured mutant strain KR101 failed to show any plasmid band (Fig. 5, lane 3), indicating the involvement of the plasmid in CM degradation. Strain KR101 was not able to utilize CM in MSM medium, and the growth of the cured mutant was completely inhibited by CM at 300 μg/ml (Fig. 6) in MSM broth.

The degradation of different insecticides in MSM cultures inoculated with cured mutant strain KR101 were investigated (Table 2). It was able to degrade 8.8 and 12.3% of chlorpyrifos at 300 and 100 μg/ml, respectively. Similar differences in the response of the strain to the other 10 insecticides were likewise observed.

Antibiotic sensitivity

The sensitivity of *Burkholderia* sp. strain KR100 and its cured mutant strain KR101 to the eight antibiotics was bioassayed by disk diffusion (Table 3). Growth-inhibiting responses varied with compound and dose tested. At 30 μg/disk, the strain was resistant to amphotericin B, polymixin B sulfate, and tetracycline and sensitive to amoxicillin and trimethoprin. The cured derivative was resistant to amphotericin B and polymixin B sulfate and sensitive to amoxicillin, chloramphenicol, trimethoprin, and vancomycine.

Discussion

In the present enrichment study, a CM degrading isolate was obtained from a Korean rice paddy soil.

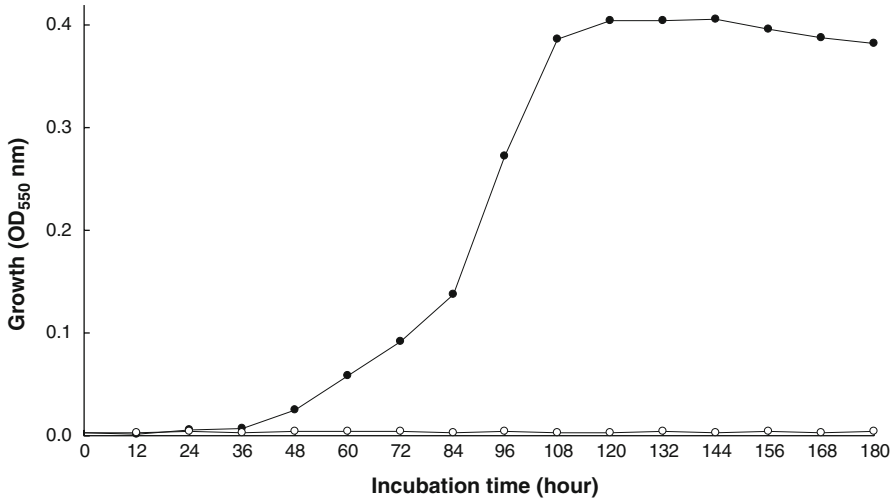

Fig. 6 Growth patterns of *Burkholderia* sp. strain KR100 (*solid circle*) and its plasmid cured mutant strain KR101 (*open circle*) in MSM supplemented with 300 μg/ml chlorpyrifos-methyl

Table 2 Degradation of different organophosphorus and carbamate insecticides by cured mutant strain KR101

Substrate	Concentration (μg/ml)	Extent of degradation (%)
Chlorpyrifos	300	8.8
	100	12.3
Diazinon	300	7.9
	100	7.2
Dicrotophos	300	1.8
	100	8.1
Dimethoate	300	2.7
	100	14.6
Fenitrothion	300	7.4
	100	6.5
Malathion	300	9.0
	100	5.7
Monocrotophos	300	8.1
	100	15.6
Parathion	300	9.6
	100	15.6
Parathion-methyl	300	7.5
	100	13.0
Carbaryl	300	10.2
	100	4.6
Furathiocarb	300	12.3
	100	16.0

Table 3 Zone diameter of growth inhibition of *Burkholderia* sp. strain KR100 and its cured mutant strain KR101 against eight antibiotics tested

Antibiotics	Zone diameter (mm) of growth inhibition					
	Strain KR100			Strain KR101		
	Dose (mg/disk)			Dose (mg/disk)		
	30	10	6	30	10	6
Amoxicillin	23	NI[a]	NI	25	20	15
Amphotericin B	NI	NI	NI	NI	NI	NI
Chloramphenicol	17	11	NI	21	17	12
Metronidazole	15	NI	NI	15	11	NI
Polymixin B sulfate	NI	NI	NI	NI	NI	NI
Tetracycline	10	NI	NI	13	10	NI
Trimethoprim	21	16	12	25	19	15
Vancomycine	16	12	12	20	16	13

[a] No inhibition

Unlike other soils, it is well-known that rice paddy soil contains largely uncharacterized microbial populations which degrade polymeric organic matter to low-molecular-weight compounds because of their peculiar characteristics, such as anaerobic condition and redox state (Khan 1982). This isolate, strain KR100, was closest to members of the *B. cepacia* group as indicated by sequence analysis of the 16S rRNA gene and taxonomical and biochemical characterizations. *B. cepacia* strains have been reported to degrade a variety of chemicals, such as 2,4,5-trichlorophenoxyacetic acid (Kilbane et al. 1983), 3-methylbenzoic acid (Higson and Focht 1992), toluene and trichloroethylene (Astrid et al. 1996), fenitrothion (Hayatsu et al. 2000), 4-nitrocatechol (Chauhan et al. 2000), and 2,4-dichlorophenoxyacetate (Poh et al. 2002).

TCP is the major degradation product of both chlorpyrifos and CM (Meikle and Youngson 1978; Tomlin 2003). TCP has been reported in soil (Singh et al. 2004), water (Liu et al. 2001), raw wastewater originating from the OP producing facility (Feng et al. 1997), and agricultural crops (Zayed et al. 2003). There have been few reports of enhanced degradation of chlorpyrifos by isolated microorganisms because of antimicrobial activity of TCP at high concentrations (Racke et al. 1990; Singh et al. 2004). TCP is low to moderately toxic to aquatic and terrestrial biota despite relatively nontoxic to mammals (Racke 1993). Although mineralization of TCP has been reported in soil (Racke and Robbins 1991), very little information exists with respect to microbial degradation of TCP. Bacterial strains capable of mineralizing TCP in the liquid medium and in soil and utilizing it as a sole source of carbon and energy have been reported in *A. faecalis* (Yang et al. 2005) and *Pseudomonas* sp. (Feng et al. 1997). In the current study, *Burkholderia* sp. strain KR100 hydrolyzed CM to TCP and dimethylthiophosphoric acid as reported previously (Meikle and Youngson 1978; Tomlin 2003). This is the first report of degradation of both CM and TCP by *Burkholderia* sp. Strain KR100 was capable of utilizing 100 μg/ml of TCP as a sole source of carbon but did not degrade TCP at 300 μg/ml. Less degradation at high concentrations of TCP might be attributed to toxic to the strain cells as stated above. Growth of *A. faecalis* and degradation of TCP was known to be not affected by TCP at concentrations of more than 800 mg/l (Yang et al. 2005), whereas soil concentrations higher than 100 mg/l of TCP retarded the microbial degradation of several insecticides (Chapman and Harris 1990). The degradation pathway of TCP by KR100 remains to be proven, although TCP degradation by the *Pseudomonas* ATCC 700113 yields CO_2, chloride, and unidentified polar metabolites (Feng et al. 1997).

The metabolic specificity of OP degrading microorganisms has been reported to be determined by the chemical resemblance among the OP compounds (Racke and Coats 1987), although certain microorganisms can degrade several compounds (Singh and Walker 2006). Substrate specificity studies demonstrated that degradation by *Burkholderia* sp. strain KR100 varied with compound and concentration used. The intact cell of the strain degraded diazinon, dicrotophos, parathion, and parathion-methyl at 100 μg/ml but not at 300 μg/ml. At 100 μg/ml, the degradation of these insecticides was more pronounced in the intact cell than the cell extract of the strain. These results suggest that high concentrations were toxic to normal growth and metabolism. Similar results were obtained by Jia et al. (2006), who found that a *Paracoccus* strain was able to tolerate and grow on 300 mg/l optimally but did not grow on a concentration higher than 500 mg/l. Additionally, the strain KR100 was able to degrade phosphorothioates, phosphorodithioates, and dimethyl phosphates, which may explain the reason for degradation of the compounds. All the insecticides tested have phosphotriester bond in molecules, indicating that the compounds were hydrolyzed at a phosphotriester bond. Due to its broad specificity against a range of OPs, strain KR100 possesses a great potential to provide a versatile gene and enzyme system that can be used for the remediation of toxic OP products.

Bacterial plasmid plays a role in the degradation of xenobiotics (Sayler et al. 1990; Singh and Walker 2006). A few of OP degrading bacteria were found to carry plasmids encoding an insecticide hydrolyzing enzyme, and the genes for the degradation of some OPs such as parathion were identified on the plasmids (Sayler et al. 1990; Singh and Walker 2006). Bacterial strains from the genera *Pseudomonas* (Serdar et al. 1982) and *Flavobacterium* (Mulbry et al. 1986) encode organophosphorus hydrolase (*opd*) genes on a 66-kb plasmid (pCMS1) and a 43-kb plasmid (pPDL2), respectively. Two or more plasmid mediated degradation of fenitrothion (Hayatsu et al. 2000) and carbaryl (Hashimoto et al. 2002) has been reported in *Burkholderia* sp. and *Rhizobium* sp., respectively. However, no data is available on the plasmid associated CM degradation by *Burkholderia* sp. strain KR100, isolated from a Korean rice paddy soil, although *Burkholderia* sp. strain NF100 has a fenitrothion degradation pathway encoded on two plasmids, pNF1 (105 kb) and pNF2 (33 kb) (Hayatsu et al. 2000). In the present study, the cured mutant strain KR101 was almost unable to utilize CM and other 11 insecticides as a sole carbon source. A single plasmid of ~50 kb in size was found to be responsible for carrying genes for CM degradation in the strain KR 100. Genes encoding resistance to amphotericin B, polymixin B sulfate, and tetracycline were also located on the plasmid. Similar results were obtained by Nawab et al. (2003), who found that a single plasmid of ~54 kb was involved in

γ-hexachlorocyclohexane (γ-HCH) degradation in two *Pseudomonas* strains and that these strains were resistant to antibiotics, such as cloxacillin, doxycycline, methicillin, and neomycin.

Results of the present study indicate that the ability to degrade high concentrations of CM and TCP could make *Burkholderia* sp. strain KR100 an ideal candidate for its application in OP degradation and bioremediation at the contaminated soil, water and crop. Biodegradation is considered to be a reliable cost-effective technique for pesticide abatement and a major factor determining the fate of OPs in the environment as suggested by Munnecke (1976). For practical use of strain KR100 as a novel bioremediation agent to proceed, further research is necessary on the ability of the strain to degrade CM and residues when induced back into the highly competitive soil environment. Other areas requiring attention are CM degrading genes and formulations for improving the degradation and bioremediation potency and for stability in soil, water, or crop.

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