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ORIGINAL PAPER

Isolation of a novel gene encoding a 3,5,6-trichloro-2-pyridinol degrading enzyme from a cow rumen metagenomic library

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Abstract 3,5,6-trichloro-2-pyridinol (TCP) is a major metabolite of the insecticide chlorpyrifos and is hazardous to human and animal health. A gene encoding a TCP degrading enzyme was cloned from a metagenomic library prepared from cow rumen. The gene (*tcp*3A) is 2.5 kb in length, encoding a protein (Tcp3A) of 599 amino acid residues. Tcp3A has a potential signal sequence, as well as a putative ATP/GTP binding site, and a likely amidation site. The molecular weight of the enzyme is 62 kDa by SDS–PAGE. Comparison of Tcp3A with the NCBI database using BLASTP revealed homology to amidohydrolase proteins. Recombinant *Escherichia coli* harboring the *tcp*3A gene could utilize TCP as the sole source of carbon. TLC and HPLC revealed that TCP was degraded by recombinant *E. coli* harboring *tcp*3A. This is the first report of a gene encoding a TCP degrading enzyme.

Keywords Chlorpyrifos · 3,5,6-trichloro-2-pyridinol · TCP degrading enzyme · Rumen metagenome

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Introduction

Chlorpyrifos is an organophosphorus insecticide, one of many used worldwide for crop protection (Zhang et al. 2002). The primary degradation product of chlorpyrifos, by both hydrolysis and photolysis, is 3,5,6-trichloro-2-pyridinol (TCP). Cleavage of a phosphordiester bond in the chlorpyrifos molecule generates TCP and diethylphosphorothioate. TCP is also a metabolic product of structurally related compounds including methyl chlorpyrifos and triclopyr. Most reports on the fate of chlorpyrifos in soil and compost fail to examine the fate of TCP (Bakiamoh

et al. 1999). Because TCP is more polar and water soluble than chlorpyrifos (Racke 1993), it is more mobile in soil and more leachable into groundwater and surface water (Manclús and Montoya (1995). Indeed, TCP is classified as persistent and mobile by the US Environmental Protection Agency with a half-life ranging from 65 to 360 days in soil (Armbrust 2001). Low levels of TCP have been detected in the tissues of farm animals treated with chlorpyrifos, including cattle (McKellar et al. 1976; Dishberger et al. 1977; Ivey 1979), sheep, hogs (Ivey and Palmer 1979) and goats (Cheng et al. 1989), as well as in fish (Marshall and Roberts 1978; Barron et al. 1991). TCP was also found in the urine of human volunteers exposed to chlorpyrifos (Davis 1977; Nolan et al. 1984), and can be used to biomonitor for exposure to chlorpyrifos (Nolan et al. 1984). In general, pesticide degradation in soil is influenced by both biotic and abiotic factors, which act in tandem and complement one another in the microenvironment (Singh et al. 2003).

There is great interest in identifying biocatalysts to aid in the degradation of toxic compounds such as chlorpyrifos and TCP. Screening of novel biocatalysts from microorganisms isolated using traditional cultivation techniques is limited because more then 99% of microbes present in various environments cannot be cultured (13). However, a vast genetic diversity of environmental microorganisms can be explored using culture-independent metagenomic approaches. The rumen ecosystem is comprised of a diverse population of obligatory anaerobic bacteria, fungi and protozoa defined by the intense selective pressures of the ruminal environment (Schloss and Handelsman 2003; Streit et al. 2004). The rumen ecosystem is comprised of a diverse population of obligatory an anaerobic bacteria, fungi and protozoa defined by the intense selective pressures of the ruminal environment (Cho and Yun 2005). The rumen microbial population presents a rich, and until recently, under-utilized source of novel enzymes. The variety of enzymes present in rumen arises from the diversity of the microbial community, and also from the multiplicity of fibrinolytic enzymes produced by individual microorganisms (Selinger et al. 1996). Previously we isolated esterase producing gene (*est*5S) from a metagenomic library of cow rumen and we isolated an esterase-encoding gene (*est*5S) from a metagenomic library of cow rumen, and expressed it in yeast (*Pichia pastoris*). The purified esterase enzyme exhibited organophosphorus pesticide hydrolytic activity (Devaiah et al. 2009). Soil organisms are a known source of chlorpyrifos and TCP degrading activities. *Alcaligenes faecalis* DSP3 (Yang et al. 2005) and *Paracoccus* sp. TRP (Xu et al. 2008), isolated from contaminated soil and activated sludge, respectively, are both able to degrade chlorpyrifos and TCP. *Burkholderia* sp. strain KR100 (Kim and Ahn 2009) was isolated based on its ability to use TCP as a sole carbon source, and can degrade chlorpyrifos as well. *Bacillus pumilus* strain C2A1 is another recently isolated soil organism that degrades both chlorpyrifos and TCP and strain C2A1 showed 90% degradation of TCP (300 mg/l) within 8 days of incubation (Anwar et al. 2009). Soil bacteria capable of degrading organochlorinated pesticides have also been reported (Hussain et al. 2007; Raina et al. 2008; Goswami and Singh 2009). The gene encoding chlorpyrifos or organophosphorus pesticide degrading enzyme was isolated previously (Fu et al. 2004; Liu et al. 2005; Zhang et al. 2006; Yang et al. 2008; Cho et al. 2009), but this work is the first report of a gene encoding a TCP degrading enzyme.

In this study, we utilized a metagenomics approach to isolate a gene encoding a TCP degrading enzyme. We constructed a cow rumen metagenomic library and screened it for recombinant bacteria expressing a TCP degrading enzyme. We identified a novel amidohydrolase gene, *tcp3*A, that encodes a TCP degrading enzyme designated Tcp3A. Moreover, we conducted biochemical characterization of Tcp3A, and provide evidence for the degradation of TCP by the recombinant bacteria harboring the *tcp*3A gene using TLC and HPLC.

Materials and methods

Chemicals and reagents

All reagents used in this study were of analytical grade and were used without further purification. 3,5,6-trichloro-2-pyridinol (TCP) was obtained from Chemservice (West Chester, PA, USA). HPLC-grade water, methanol, acetonitrile, and glacial acetic acid were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Bacterial strains and growth conditions

Escherichia coli DH5α, BL21, EPI300, and recombinant *E. coli* cells were cultured in LB (Difco) containing appropriate antibiotics (ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 12.5 μg/ml) at 37°C. TCP was used in solid and liquid growth media at 100 mg/l.

Construction of cow rumen metagenomic library and isolation of a gene encoding TCP degrading enzyme

Sampling of rumen and rumen metagenomic library construction was done as per Cho and Yun (2009). Total genomic DNA from cow rumen was sheared into approximately 40-kb fragments using a syringe needle, size-fractionated on a 5–40% linear sucrose gradient and then end-repaired to yield blunt, 5′-phosphorylated ends. The resulting DNA fragments were ligated with the cloning-ready pCC1FOS vector, and then packaged using a lambda DNA packing kit (Epicentre, USA), and transformed into *E. coli* DH5α. The resulting library was screened for clones that were able of degrading TCP by growth in M9 medium containing TCP as the only source of carbon at 30°C. The enrichment culture was serially diluted and aliquots were spread on M9 agar plates containing TCP. Approximately 200 colonies were randomly chosen and tested for their TCP degrading activity by inoculation into M9 medium containing TCP. Cultures were incubated for 10 days. The isolates showing growth on TCP were streaked onto fresh TCP-containing M9 agar plates. One positive clone was identified (pFCKY54, 40 kb insert). *Sau*3AI-digested fosmid DNA fragments (3–5 kb) of pFCKY54 were cloned into the pBluescript II SK + vector and then screened in *E. coli* DH5α for growth on TCP. One positive subclone of pBluescript II SK+, which had a 2.5-kb insert, was further characterized.

Cloning and DNA sequencing of *tcp*3A gene

For high expression of *tcp*3A, a *tcp*3A PCR product was generated with primers 5′-AATATGAATTCA TGTTTTATCCTAAAATA (sense, 2295F with *Eco*RI underlined) and 5′-TACAAGCTTATTGAGA AGACCTTCTTA (antisense, 2296R with *Hin*d III underlined) using the pBluescript II SK+ *tcp*3A clone as template. The PCR product was cloned into the expression vector pET-28a (Novagen) using *Eco*RI and *Hin*d III sites, which results in the addition of a C-terminal $(His)_6$ tag to the Tcp3A protien. The resulting plasmid was designated pET-28a(+)/*tcp*3A. Nucleotide sequences were determined by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp. USA). The nucleotide sequence data for *tcp*3A were deposited in NCBI GenBank under accession number GQ451605. Standard procedures for restriction enzyme digestion, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation and other cloning techniques were followed as described by Sambrook and Russel (2001). BLAST was used to find the protein coding regions.

Expression and purification of enzyme

E. coli strain BL21 carrying pET-28a(+)/*tcp*3A was grown at 37°C to mid-log phase in LB medium containing kanamycin (50 μg/ml). Expression was then induced by addition of 0.5 mM IPTG, and growth was continued for 12 h. The cells were harvested by centrifugation (6,000×*g*, 10 min) and washed twice with 10 mM Tris–HCl buffer (pH 7.0). The cells were resuspended in the same buffer and stored at −20°C. The frozen cells were mixed with 50 mM Tris–HCl buffer (pH 7.5) containing 1 mg of bovine DNase I (Sigma) and incubated at 37°C for 30 min. Triton X-100 was added to the suspension at a final concentration of 2.5%. The supernatant was collected and stored at 4°C. The solubilized recombinant Tcp3A with His-tag (Tcp3A-His) was purified using a HisTrap kit (Amersham). Tcp3A-His was eluted with 100 mM imidazole with 0.1% Triton X-100. The purified enzyme samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The active fractions were combined and purified by ion exchange chromatography on a Q-Sepharose column (Pharmacia), followed by dialysis. For the final purification step, the dialyzed sample was loaded onto an anion exchange Mono Q HR (Pharmacia) column pre-equilibrated with 20 mM MOPS buffer (pH 6.5). The purity of Tcp3A enzyme was assessed by SDS–PAGE. The protein concentration was determined by the method of Bradford (1976).

Growth and degradation of TCP

Degradation of TCP was measured according to Xu et al. (2008), Kim and Ahn (2009), and Anwar et al. (2009). Growth of *E. coli* DH5α (harboring *tcp*3A gene) and *E. coli* DH5α (control) using TCP as sole carbon source were quantitatively compared to assess the TCP-degradation activity of Tcp3A. Aliquots (100 µl) of suspensions of *E. coli* DH5α (harboring *tcp*3A gene) and *E. coli* DH5α (control) at OD_{600} of 0.6 were inoculated in triplicate into 50 ml of M9 medium with TCP as the sole carbon source. Six milliliter sample with three replications were collected from each flask at 0, 3, 6 and 9 days after inoculation, and the OD_{600} was measured by spectrophotometer (Thermo systems, USA). These cultures were also used as the source of material for thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Samples were centrifuged to remove the bacterial biomass prior to TLC and HPLC analysis as described below.

TLC and HPLC analysis for studying TCP degradation

Five milliliter sample was centrifuged and 1 and 4 ml of supernatant was mixed with methanol and ethyl acetate and vortexed for 30 s for HPLC and TLC analysis, respectively. Samples from the growth curve experiment described in Fig. 5 were centrifuged to remove bacterial biomass and 1 ml (for HPLC) and 4 ml (for TLC) of supernatant was mixed with methanol and ethyl acetate and vortexed for 30 s. For TLC, the ethyl acetate extracted samples were dried under vacuum using a rotary evaporator, and finally dissolved in 100 µL of HPLC grade-methanol. Ten microliter of the preparation was then spotted onto a pre-coated silica gel aluminium plate (0.25 mm, Merck, Germany). The solvent system used for detection of TCP consisted of ethyl acetate/isopropanol/ammonia water (45:30:20 v/v). The samples were run for 15 min, dried at 55°C and detected using UV light (254 nm). TCP was confirmed as the spot with approximately 0.66 of *Rf* value. For HPLC analysis, samples were dissolved in methanol and filtered through a 0.45 µm Minipore PVDF filter (Schleicher & Schuell, GmbH, Dassel, Germany). Samples were analyzed on a C18 column (250 × 4.6 mm, 5 µm, Phenomenx, CA, USA) by HPLC (Perkin- Elmer 200 series, Norwalk, CT, USA). Samples were eluted at 1 ml/min flow rate using 0.5% acetic acid:methanol (20:80) at 30°C. A 10 µl aliquot of the sample was injected onto the column. The absorbance of the separated organic acids was measured at 214 nm and quantified using TCP standard stock solutions by UV detector (Perkin-Elmer 200 series, Norwalk, CT, USA).

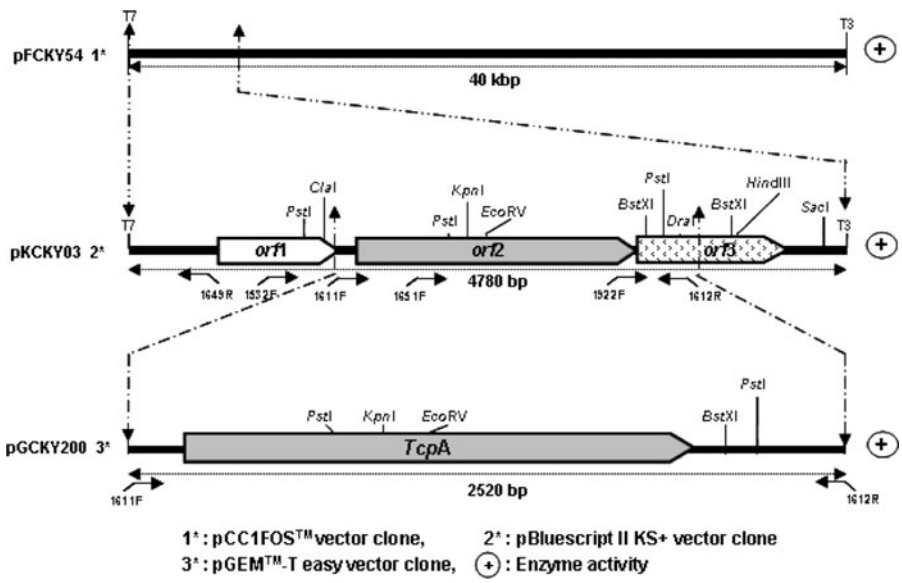

Fig. 1 Physical map of recombinant DNA pFCKY54, pKCKY03, and pGCKY200. The ORFs are shown by *thick arrows*. pKCKY03 was constructed by cloning a 4.5 kb fragment of fosmid DNA (pFCKY54) into the pBluescript II KS+ vector and pGCKY200 was constructed by cloning a 2.5 kb fragment of pBluescript II KS + DNA (pKCKY03) into the pGEM-T easy vector. The cleavage sites of restriction enzymes *Pst*I, *Kpn*I, *Eco*RV, *Bst*XI and *Pst*I are shown

Results

Cloning and sequencing of *tcp*3A

A clone that allowed *E. coli* to grow on TCP as the sole carbon source was isolated from a cow rumen metagenomic library. Both strands of the 2.5-kb insert were sequenced and the 1799 bp ORF, encoding a protein of 599 amino acids, was named *tcp*3A (Figs. 1 and 2). The calculated pI of Tcp3A is 5.45.

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1     GGATGGATGAAACGGGTCAAAACTGTTCAAATCAGCAATAATAATAACCAAATT
61    TCTTTAAGCGACAGACATTTGGGGTAAAGCTATTATCGGGAATTTGGTTATCTTTGCAAA
121   ACCAAATATGACATTTGAAAACCTTATAATATGTTTTATCCTAAAATAATAGTAATTATG
1                                   M  F  Y  P  K  I  I  V  I  M
181   AGAAAAATGAAAAAATGGATGTTTGSCGCTATCTTGATTTGTGGCACAAGTGTATTCACG
11    R  K  M  K  K  W  M  F  X  A  I  L  I  C  G  T  S  V  F  T
                    Signal peptide
241   GCGTGCTCGTCGAATGAAGACAACCCCAGTAAGGGAGACACCCCTTCAGAAAAAAACATT
31    A  C  S  S  N  E  D  N  P  S  K  G  D  T  P  S  E  K  N  I
301   GCCGATCTCGTGGTCTATGGTAAGATTTTCACTTCCGAAGGCAATCAGGTGGTGGAAGCC
51    A  D  L  V  V  Y  G  K  I  F  T  S  E  G  N  Q  V  V  E  A
361   TTTGCCGTGAAGGACGGCAAGTACATCTATGTAGGCGACAAGACGGGAGCCGAAGCCTAT
71    F  A  V  K  D  G  K  Y  I  Y  V  G  D  K  T  G  A  E  A  Y
421   GTCGAGGCAGGCAAGACCGAGGTGGTGGACTATACCGGCAAGGGACTTGTAATGCCCGGT
91    V  E  A  G  K  T  E  V  V  D  Y  T  G  K  G  L  V  M  P  G
      ATP/GTP binding site
481   TGCGGCAACGGACATGCACACTACTCGATGGCTAACGCCATCAAATTAGTCGGTGCGACA
111   C  G  N  G  H  A  H  Y  S  M  A  N  A  I  K  L  V  G  A  T
541   GTAGGATTAGAGGACAATGCCAGCAAGTTCCTGACGGAAATCGTACCTGCTGCTGTCAAG
131   V  G  L  E  D  N  A  S  K  F  L  T  E  I  V  P  A  A  V  K
601   CAGGCAAGAGACGCAGGGGCAAAAAGTATCTTTGGTTTCGGATGGAATGTCATGTCTTCG
151   Q  A  R  D  A  G  A  K  S  I  F  G  F  G  W  N  V  M  S  S
661   AGGGTAATATGCCTACTCGCCAACAGTTGGATGCCATCTGCAGCGATATTCCTATGTACT
171   R  V  I  C  L  L  A  N  S  W  M  P  S  A  A  I  F  L  C  T
721   TCTCAGACAATGAGGGTCACAAGGGGCTGGCAAATACCGCCTTACTGGTCAAGGCCGGTA
191   S  Q  T  M  R  V  T  R  G  W  Q  I  P  P  Y  W  S  R  P  V
781   TTAAAGGCTGACGGCACCGTGCTCAAAAAAGATGGAGACATATTAGGTGGTGAGATTGTA
211   L  K  A  D  G  T  V  L  K  K  D  G  D  I  L  G  G  E  I  V
841   ATGGGTACCGACGGCACACCTACAGGCTATCTGAAGGAGCAGGCCGGCACTTACGCACGT
231   M  G  T  D  G  T  P  T  G  Y  L  K  E  Q  A  G  T  Y  A  R
901   TCCTTCTTAGACAATGAAAGTCTCTTTAGCCTTGACATTGCAAGTGATATCTTGCCAAAA
251   S  F  L  D  N  E  S  L  F  S  L  D  I  A  S  D  I  L  P  K
961   ATCGAGCAGCAGCTATTGTCTGAGGGCTACACCATGTATCTCGATGGCTGGGGTTCCTAT
271   I  E  Q  Q  L  L  S  E  G  Y  T  M  Y  L  D  G  W  G  S  Y
1021  TTCTTTAACACTAATTTCTATCAGGCCGCTCAACAGTTAGACAATGCTGGTGGTATGCAC
291   F  F  N  T  N  F  Y  Q  A  A  Q  Q  L  D  N  A  G  G  M  H
1081  TTCGTGTTGGGCCTGTCTTACGAGATTGAAAGTTGGATGAATATTGACGAAGCCCTGCCC
311   F  V  L  G  L  S  Y  E  I  E  S  W  M  N  I  D  E  A  L  P
1141  AAGGCTGCTGACGCCAAGAAATTTGCTTCCACTCGCGTCAAACCGAACTGGATTAAACTC
331   K  A  A  D  A  K  K  F  A  S  T  R  V  K  P  N  W  I  K  L
1201  TTTATGGACGGCACGGTGGAGACTGGCACCGGATTTGGAATCCGCTCTATCCTGACGGAC
351   F  M  D  G  T  V  E  T  G  T  G  F  G  I  R  S  I  L  T  D
1261  ATCAGGGCCTGCCCAACCTGGTCGGAAGAGCAACTGACCGACATGACACGTGTGGCTAAT
371   I  R  A  C  P  T  W  S  E  E  Q  L  T  D  M  T  R  V  A  N
1321  GAAAACGGTGTAACTATGCACGTACATGTCATGGGCAACAAGGGCGTCACGCGTATTGTC
391   E  N  G  V  T  M  H  V  H  V  M  G  N  K  G  V  T  R  I  V
1381  AACGCCTTCATCAATGGCGGAAAGGATGAGATGCGTAACACGCTGGTTCACGTGCGTAAT
411   N  A  F  I  N  G  G  K  D  E  M  R  N  T  L  V  H  V  R  N
1441  ATTGACGAAGCAGATTACAAGCGTATGGCAGACCATAATATCTATGTCACCTCAGGTGTG
431   I  D  E  A  D  Y  K  R  M  A  D  H  N  I  Y  V  T  S  G  V
1501  CTTTGGCATTACGGTCCTGATGGTCTGGCTGAGTATATGCGCGAGCATGGTATCGTTCCG
451   L  W  H  Y  G  P  D  G  L  A  E  Y  M  R  E  H  G  I  V  P
1561  GCAGGCCAGGAAGACAAATCTTATCCGATGAAATCGTATTTTGACAACGGCATTATTGTG
471   A  G  Q  E  D  K  S  Y  P  M  K  S  Y  F  D  N  G  I  I  V
1621  ACCACCCACTCGGACTATCCTGCACTGAGCGGCAGCCCAGACGACCCGTTTGGCATTATG
491   T  T  H  S  D  Y  P  A  L  S  G  S  P  D  D  P  F  G  I  M
1681  GAGATTGCCGTGACAGGTATGCTTACAGGAGTGAACGGAAAGCCCTACTGGACTGAAGAA
511   E  I  A  V  T  G  M  L  T  G  V  N  G  K  P  Y  W  T  E  E
1741  CTCGTCACCCGCGAGCAGGCCATCGCCGCAATGACCATTAACATTGCCAAGCAGATGTTC
531   L  V  T  R  E  Q  A  I  A  A  M  T  I  N  I  A  K  Q  M  F
1801  ATCGAGAATGAGCGTGGCAGCATCAAGACAGGCAAGTATGCCGACTTCCTCCTCGTCACC
551   I  E  N  E  R  G  S  I  K  T  G  K  Y  A  D  F  L  L  V  T
1861  AAGGATGTGCTTTCGTGTCCTGAGAATGAGATTCACGAGGCCAAGCCCGCAGCCACCTAC
571   K  D  V  L  S  C  P  E  N  E  I  H  E  A  K  P  A  A  T  Y
1921  TTCGAGGGTAAGAAGGTCTTCTCAATGTAACATAGTATAAAAGGACAACAATATGAAAGA
591   F  E  G  K  K  V  F  S  M  *
            Amidation site
1981  GATTATAGACTATTCCAAGAAGGAAAACTGGTATCAGATTCCAGAGATTACCAAGGACGT
2041  GGACACATTCTACATCTATGCAACGGAGTACATTCTGAAAAGTTTCGATGAAGGCGCACC
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Fig. 2 Nucleotide sequence and deduced amino acid sequence of *tcp*3A gene from rumen metagenome. Signal peptide between 1 and 30 positions, ATP/GTP binding site between 89 and 97 positions and amidation site between 593 and 595 positions

The *tcp*3A gene was amplified using PCR, cloned into pET28a(+) vector, and transformed into *E. coli* strain BL21 for expression.

Amino acid sequence analysis of Tcp3A

BLAST analysis comparing the Tcp3A amino acid sequence with the NCBI database revealed significant homology with amidohydrolase proteins, including the amidohydrolase of *Mycobacterium smegmatis* MC2 (25% identity), exoenzyme regulatory protein of *Pectobacterium* sp. SCRI1043 (25% identity), amidohydrolase 3 of *Mycobacterium* sp. MCS (26% identity), and urease domain protein of *Microscilla marina* ATCC 23134 (24% identity) (Fig. 3). Analysis of the amino acid sequence with the program PSORT revealed a potential signal sequence from aa 1–32, an ATP/GTP binding site between aa 89-97, and an amidation site from aa 593–595 (Fig. 2).

Purification and characterization of Tcp3A

Tcp3A protein was purified from *E. coli* BL21 harboring the pET28a(+)/*tcp*3A plasmid using column filtration techniques (see Materials and methods). Protein fractions were analyzed by SDS–PAGE. Induction of *tcp*3A gene expression with IPTG produced a distinct protein band of 62 kDa (Fig. 4).

Cell growth and TCP degradation in liquid culture

The ability of *E. coli* DH5α harboring *tcp*3A to degrade TCP was studied in M9 medium containing TCP. *E. coli* DH5α harboring *tcp*3A was able to use TCP as the sole source of carbon for its growth (Fig. 5). *E. coli* DH5α harboring *tcp*3A degraded TCP very quickly till 3 days of inoculation, and after that degraded slowly till the ninth day of growth. Both TLC and HPLC analysis revealed that TCP was degraded by *E. coli* DH5α harboring *tcp*3A and HPLC analysis also indicated that the concentration of TCP in the cell supernatant decreased from 0 day to the 9th day of growth (Fig. 6).

Discussion

In a previous study, we isolated the *est*5S gene from a cow rumen metagenomic library, and demonstrated

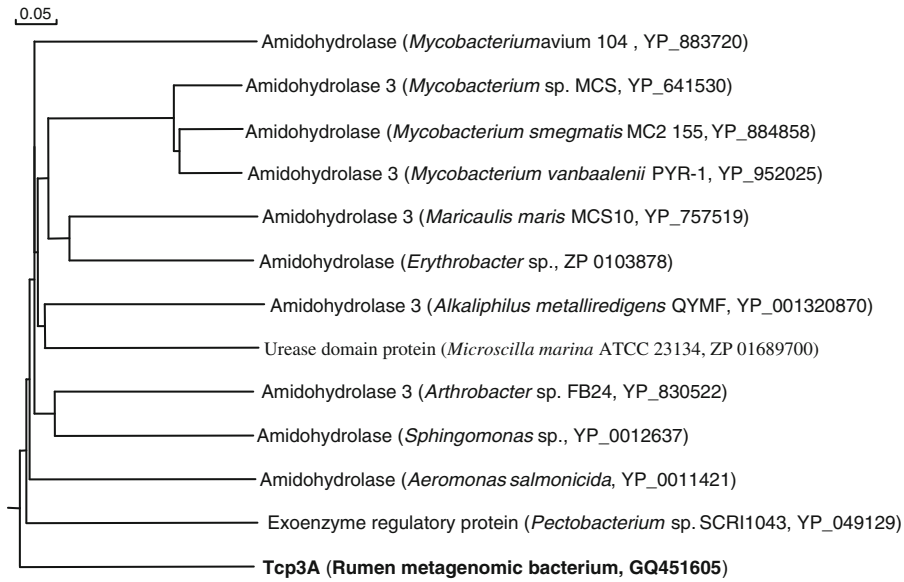

Fig. 3 Phylogenetic placement of TCP degrading gene (*tcp*3A). Bacterial strains and accession number within the parentheses

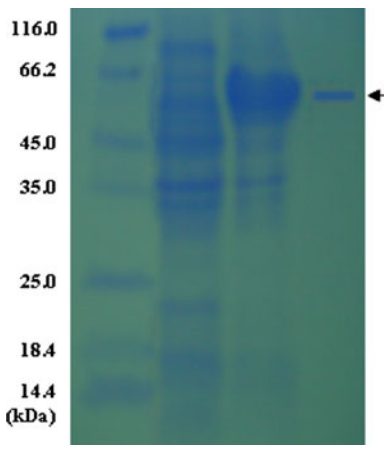

Fig. 4 Electrophoretic analysis of the purified Tcp3A Separation was performed on a 12.5% (W/V) SDS polyacrylamide gel. The gel was stained with Coomassie *blue* R-250. *Lane 1*: Molecular weight markers; *lane 2*: crude extract from *E. coli* BL21 containing pET-28a/*tcp*3A; *lane 3*: crude extract from IPTG-induced *E. coli* BL21 containing pET-28a/*tcp*3A; *lane 4*: purified Tcp3A from Hi-Trap kit (Amersham)

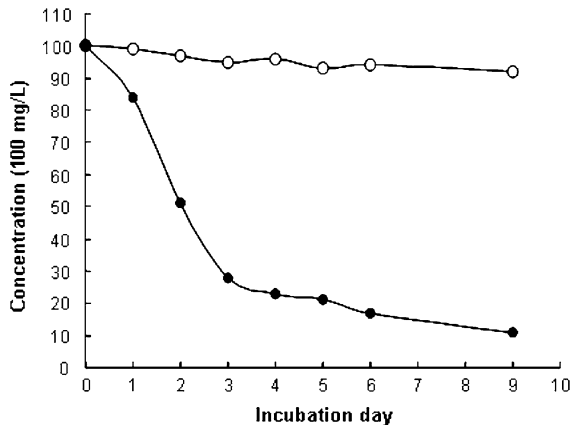

Fig. 5 Degradation of TCP by *E. coli* DH5α and recombinant *E. coli* DH5α harboring TCP degrading gene (*tcp*3A) in M9 medium containing TCP (100 mg/l). Concentration of TCP was measured by HPLC (Perkin- Elmer 200 series, Norwalk, CT, USA) using a C18 column (250 × 4.6 mm, 5 µm, Phenomenx, CA, USA)

that it was capable of degrading organophosphorus pesticides including chlorpyrifos (Devaiah et al. 2009). Chlorpyrifos and/or organophosphorus pesticide degrading genes have been isolated from a number of cultured bacterial strains including *Sphingomonas* sp. Dsp-2 (Fu et al. 2004), *Pseudomonas* sp. WBC-3 (Liu et al. 2005), *Burkholderia* sp. FDS-1 (Zhang et al. 2006), *Stenotrophomonas* sp. YC-1 (Yang et al. 2008), and lactic acid bacteria (Cho et al. 2009). Degradation of organophosphorus pesticides by cultured microorganisms, even at high pesticide concentration, has been reported previously (Liu et al. 2005). In most cases described to date, aerobic bacteria tend to transform chlorpyrifos by hydrolysis to produce diethylthiophosphoric acid

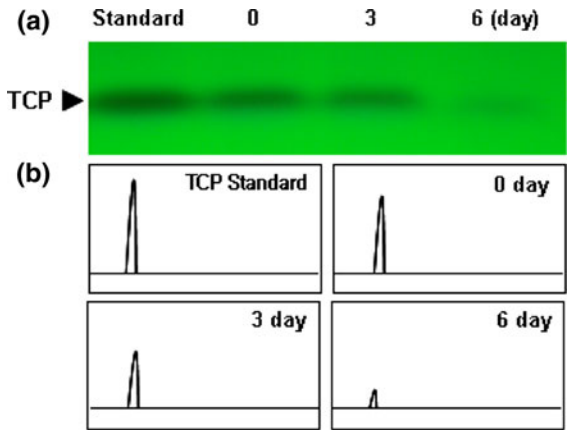

Fig. 6 Degradation of TCP by *E. coli* DH5α harboring TCP degrading gene (*tcp*3A). **a** TLC, and **b** HPLC result

(DETP) and TCP (Cook et al. 1980). TCP is a major metabolite in environments where chlorpyrifos or triclopyr has been applied. Singh and Walker (2006) described microbial metabolism of TCP by a reductive dechlorination pathway (Feng et al. 1997). In this pathway, TCP is first reductively dechlorinated into chlorodihydro-2-pyridone, which is further dechlorinated to tetra-hydro-2-pyridone, and ring cleavage of this compound result in formation of maleamide semialdehyde, which is metabolized to water, carbon dioxide, and ammonium ions. Bacterial strains capable of TCP degradation have been isolated including *Alcaligenes faecalis* DSP3 from contaminated soils around a chemical factory (Yang et al. 2005), *Paracoccus* sp. TRP from activated sludge (Xu et al. 2008), *Burkholderia* sp. KR100 from paddy soil (Kim and Ahn 2009) and *Bacillus pumilus* C2A1 from soil (Anwar et al. 2009). However, a gene required for TCP degradation had not been cloned until this work.

In this study, we utilized a metagenomic approach to isolate a gene encoding a TCP degrading enzyme from cow rumen. We constructed a cow rumen metagenomic library in a fosmid vector and screened it for TCP degrading clones. Fosmids are good vectors for constructing metagenomic libraries because of their high cloning efficiency, improved stability in *E. coli*, and large insert size (Cho and Yun 2005). Cloning efficiency is an important factor for construction of a metagenomic library because of the low frequency of finding desirable genes from a library of diverse microbial genomes (Struthers et al. 1998; Karpouzas and Walker 2000). Environmental DNA libraries containing several hundred thousand clones with small inserts can require screening of a large number of clones (Racke 1993; Zhang et al. 2002). However, our approach of library preparation in a fosmid and subsequent subcloning was very effective in searching for a TCP degradation gene (*tcp*3A).

The *tcp*3A gene is 1.7 kb, with a predicted signal peptide from aa 1 to 32, including a transmembrane region from aa 15 to 35. Tcp3A is predicted to be an extracellular protein with a size of 62 kD. The *tcp*3A gene was cloned in pET-28a(+) and expressed in *E. coli* BL21. The molecular weight of the purified protein was consistent with the predicted molecular weight. One unique feature of Tcp3A is that it contains an amidation signal at aa 592–595. BLAST analysis comparing the Tcp3A amino acid sequence with the NCBI database revealed significant homology with amidohydrolase proteins. Amidohydrolases or amidases can select and attack amide bonds (Hassal 1990). So Tcp3A enzyme supposed to attacks the terminal amide bond in the structure of TCP (Mauriz et al. 2007).

The TCP degradation activity of Tcp3A was examined using both TLC and HPLC analysis of the supernatants of Tcp3A-expressing *E. coli* during growth on TCP as the sole carbon source. TLC studies revealed that TCP was being degraded by the recombinant bacteria starting from 2 days of incubation in M9 media with TCP. HPLC studies revealed that some TCP was still detectable after 9 days of growth in the M9 media with TCP. Others have observed degradation of TCP by bacteria growing in culture. Xu et al. (2008) observed TCP degradation by *Paracoccus* sp. strain TRP. Kim and Ahn (2009) isolated *Burkholderia* sp. strain KR100, which utilized TCP as the sole source of carbon for its growth, and recently Anwar et al. (2009) observed that *Bacillus pumilus* strain C2A1 showed 90% degradation of TCP (300 mg/l) within 8 days of incubation. TCP-degrading bacteria have been somewhat difficult to isolate, probably due to the antimicrobial activity of TCP at higher concentrations because TCP contains three chlorine atoms on the pyridinol ring, and free chlorine has toxic effects on the microorganisms (52). Racke et al. (1990) suggested that the accumulation of TCP in chlorpyrifos-treated soil acts as a buffer and prevents the proliferation of chlorpyrifos-degrading microorganisms. TCP contains three

chlorine atoms on the pyridinol ring and free chlorine has toxic effects on the microorganisms (Feng et al. 1997). However, growth of the Tcp3A-expressing *E. coli* was not affected by TCP concentrations up to 100 mg/l in liquid culture. This is the first report of an enzyme that can degrade TCP, the major metabolite of chlorpyrifos. The Tcp3A enzyme may facilitate development of pathways for biocatalytic degradation of organophosphorus pesticides. Further research works are need to carried out to investigate the mechanism of TCP degradation by Tcp3A enzyme.

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