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Identification of putative Cof-like hydrolase associated with dehalogenase in *Enterobacter cloacae* MN1 isolated from the contaminated sea-side area of the Philippines

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ABSTRACT

Aims: The present study aimed at molecular identification of putative Cof-like hydrolase associated with dehalogenase gene from a bacterium that was isolated from a contaminated sea-side area in the Philippines. The bacterium was subjected to 16S rRNA gene sequence analysis for identity of the genus and species.
Methodology and results: Based on basic microbiological analysis and 16S rRNA sequence determination, strain MN1 showed high sequence identity to *Enterobacter cloacae*. This is the first reported study that *Enterobacter* could degrade 2,2-dichloropropionate (2,2-DCP). A putative dehalogenase gene like was identified by direct sequencing and analysis of the PCR-amplified genomic DNA of the bacterium. A comparative analysis of the sequence data revealed that the amino acid sequence is closely related to several Cof-like hydrolase associated with L-specific dehalogenases.
Conclusion, significance and impact of study: Current study may suggest that the hydrolase may have similar function to dehalogenase. However, further analysis like enzyme assay need to be carried out to confirm this. Putative dehalogenase gene can be amplified using PCR technique provided that the specific primers designed were used.

Keywords: Enterobacter cloacae MN1, pollutant degradation, Cof-like hydrolase, dehalogenase

INTRODUCTION

Halogenated organic compounds are found widely throughout the environment. Microbial catabolic enzymes involved in the conversion of organohalogen compounds have potential applications in environmental technologies and the chemical industry (Mowafy *et al.*, 2010; Kurihara, 2011; Mutarasaiah *et al.*, 2012). For the catabolism of halogenated organic compounds in the biosphere, dehalogenation is regarded as the key first step (Hill *et al.*, 1999). The use of dehalogenases in industrial processes is well established and this enzyme is also useful in environmental technology to decontaminate environment polluted with harmful halogenated compounds such as man-made chemicals used as refrigerants, fire retardants, paints, solvents, herbicides and pesticides (Fetzner and Lingens, 1994; Huyop and Nemati, 2010).

The dehalogenase enzyme belongs to the family of hydrolases that specifically act on halide bonds in carbon-halide compounds. Other names given to this enzyme are: haloacid dehalogenase (HAD), 2-haloacid dehalogenase, 2-haloacid halidohydrolase, 2-haloalkanoic acid dehalogenase, 2-haloalkanoid acid halidohydrolase, 2-halocarboxylic acid dehalogenase II, DL-2-haloacid dehalogenase, D- or L-2-haloacid dehalogenase and L-DEX (Nardi-dei *et al*., 1997). So far, Cof-like hydrolases is also categorized in the same family and commonly found in many organisms. Therefore, Cof-like hydrolase maybe distantly related to dehalogenase (Ren *et al*., 2010).

Previously, a group of bacteria that can grow on halogenated compound as sole source of carbon was identified and the corresponding genes were isolated (Thomas *et al*., 1992; Cairns *et al*., 1996; Fortin *et al*., 1998; Jing and Huyop, 2007; Jing *et al*., 2008; Ismail *et al*., 2008; Mesri *et al*., 2009; Zulkifly *et al.*, 2010; Hamid *et al*., 2010 a, b). However, the discovery of new dehalogenases is still the highlighted area of research and deserves further study (Yusn and Huyop, 2009).

In the current study, we have isolated a bacterium from contaminated sea-side in the Philippines. The strain

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was grown effectively on selective minimal medium containing 2,2-DCP as a sole source of carbon and energy. Furthermore, we identified a Cof-like hydrolase gene presumably responsible for bacterial growth in 2,2-DCP. The bacterium was subjected to 16S rRNA for analysis for genus and species identification.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from the seaside area of Tigbauan, Iloilo, Philippines. This area is highly polluted with domestic waste and fisheries industry products discharge.

Medium and culture condition

A distinctive dehalogenating bacterium was grown at 37 °C for 3 to 4 days on a rotary shaker at 150 rpm in 250 mL flasks containing 100 mL minimal medium. The liquid PJC minimal media was prepared as 10X concentrated basal salts containing $K_2HPO_4 \cdot 3H_2O$ (42.5g/L), $NaH_2PO_4 \cdot 2H_2O$ (10.0 g/L) and $(NH_4)_2SO_4$ (25.0 g/L). The trace metal salts solution was a 10X concentrate that contained nitriloacetic acid (NTA) (1.0 g/L), $MgSO_4$ (2.0 g/L), $FeSO_4 \cdot 7H_2O$ (120.0 mg/L), $MnSO_4 \cdot 4H_2O$ (30.0 mg/L), $ZnSO_4 \cdot H_2O$ (30 mg/L) and $CoCl_2$ (10.0 mg/L) in distilled water (Hareland *et al.*, 1975).

Minimal media for growing bacteria contained 10 mL of 10X basal salts and 10 mL of 10X trace metal salts per 100 mL of distilled water. The carbon source 2,2-dichloropropionic acid (2,2-DCP) was neutralized with NaOH, filter sterilized and added to the autoclaved medium to a final concentration of 20 mM. The growth was determined by measuring the absorbance at A_{600nm} and the release of chloride ions at A_{460nm} as described before (Jing and Huyop, 2007). The basic properties of the isolated strain were characterized using standard microbiological techniques (Nemati, 2012).

PCR amplification of 16S rRNA gene and analysis

PCR was carried out to amplify the 16S rRNA gene and the universal primers were Fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP1 (5'-ACG GTC ACC TTG TTA CGA CTT-3') (Fulton and Cooper, 2005). DNA *Taq* polymerase was used along with the buffer supplied by the manufacturer (Promega). Universal 16S rRNA forward and reverse primers were synthesized by 1st BASE Laboratory Malaysia Sdn. Bhd..

The amplification reactions contained in 50 µL with 300 ng template DNA, 20 pmol forward primer (Fd1), 20 pmol of reverse primer (rP1), 25 µL (2X) PCR master mix (Fermentas Inc. USA) and deionized water. PCR cycle was set as: initial denaturation 94 °C for 5 min, followed by cooling, denaturation 94 °C and annealing 55 °C for 1 min, extension 74 °C for 4 min and final extension 74 °C for 10 min. The PCR product was electrophoresed on a 1% agarose gel. For sequencing reaction, the PCR product was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) prior sending for sequencing (1st Base Laboratory, Malaysia).

The sequences were analysed by sequence comparison in the public databases using BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The neighbour-joining phylogenetic tree was constructed using MEGA5 software (Tamura *et al*., 2011).

DNA extraction, PCR and analysis of the PCR product

The genomic DNA was extracted from pure culture isolates by a standard technique (Wizard genomic purification kit-Promega). The PCR primers were designed using Primer 3 program version 0.4.0 (Rozen and Skaletsky, 2000). PCR amplification was performed using primers a) Forward 5´ GTG GCA ACC ACA GGC TAT CT 3´and b) Reverse 5´ GGA AGG CAT GTT TGT GCT CT 3´ (Nemati, 2012). These primers were designed based on haloacid dehalogenase like hydrolases-HAD superfamily (i.e. L-2-haloacid dehalogenase).

The PCR conditions were set at 30 cycles of the following parameters: denaturation 95 °C for 1 min, annealing 55 °C for 1 min, extension 72 °C for 2 min. The PCR product was purified using *ExoSAP*-IT PCR Clean-up Kit (GE Healthcare Bio-Sciences Corp. USA) for sequencing at 1st Base Laboratory, Malaysia. The DNA sequencing results were analysed and converted into amino acids. The amino acid sequence was BLASTp in public database of NCBI.

RESULTS AND DISCUSSION

Morphology and biochemical analysis

A bacterial strain utilizing 2,2-DCP as a sole carbon source was isolated. Preliminary experiment suggested that the isolated strain belongs to the *Enterobacteriacae* species (Table 1). The cells showed good growth in liquid minimal medium at 37 °C using 20 mM 2,2-DCP as the sole source of energy with a doubling time of 10 h.

Table 1: Properties of the isolated strain.

Properties	Characterization
Colony morphology	Raised edge and round
Pigmentation	Yellowish to white
Gram staining	Negative
Physical morphology	Rod-shaped
Size	0.5 - 1 µm
Motility	+
Spore staining	+

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Table 2: List of high similarity 16S rRNA obtained from BLASTn.

Closest genus/species names	Accession no.	Identity	*e*-value
Enterobacter cloacae E717	EF059865.1	99%	0.0
Enterobacter cloacae ATC13047	CP001918.1	99%	0.0
Enterobacter cloacae 279-56	NR028912.1	99%	0.0
Enterobacter cloacae FR	EU849019.1	99%	0.0
Enterobacter cloacae SJ 6	EU779827.1	99%	0.0
Enterobacter cloacae SDM	HQ434623.1	99%	0.0
Enterobacter cloacae LCR70	FJ976579.1	99%	0.0
Enterobacter sp. BSRA2	FJ868806.1	99%	0.0
Enterobacter sp. BSRA3	FJ868807.1	99%	0.0

Identification of the strain using 16S rRNA gene analysis

Current species was identified as *Enterobacter cloacae* based on 16S rRNA gene analysis. The gene sequence was analysed and compared to the sequence in the GenBank using BLASTn analysis tool. High similarity of 16S rRNA sequences was shown in Table 2. The result showed 99% identity to those of the *E. cloacae*. Therefore, the bacterium was designated as *E. cloacae* MN1.

Analysis of putative Cof like gene and protein analysis

A PCR fragment of the expected size (1.8 kb) was generated and the fragment was sequenced. The deduced amino acid sequence revealed a single open reading frame (ORF) encoding 301 amino acids that starts from the first ATG codon at 46th nucleotide and stop at 939th (Figure 1). Furthermore, during the amino acid sequence analysis, HAD-domain has been identified obviously which is a perfect confirmation of the responsibility of the gene in producing of L-haloacid dehalogenase. The second ATG codon at position 70 might act as an alternative initiation site. The BLASTp search of the deduced amino acid suggested 85% sequence homology to Cof like hydrolases. Other proteins that matched were HAD-superfamily hydrolase from *E. cloacae* NCTC 9394 (76%), Cof like hydrolase from *Enterobacter* sp. 638 (70%), Cof like hydrolase from *Escherichia coli* ATCC 8739 (62%), HAD family hydrolase from *E. coli* 536 (62%), Cof like hydrolase from *Shigella flexneri* VA-6 (62%), Cof like hydrolase from *Klebsiella variicola* At-22 (62%), Cof like hydrolase from *Pantoea* sp. At-9b (42%), and Cof like hydrolase from *Shigella sonnei* 53G (61%).

Evolutionary relationship of the *Enterobacter cloacae* MN1 putative Cof-like hydrolase with other related dehalogenase sequences

A phylogenetic tree was constructed to infer molecular analysis of evolutionary relationship (Figure 2). Current study revealed Cof-like hydrolase in MN1 had minimal relationship with conventional dehalogenases from various genuses. However, HAD-L from thermophilus *Sulfolobus tokodaii* strain 7 was placed in the same clade with the current amino acid sequence suggesting that the dehalogenase has a joint ancestor with it.

On the basis of phenotypic and genotypic characters and also studies of the basic properties and the 16S rRNA sequence analysis, MN1 is closely related to more than 9 types of *E. cloacae*. Therefore, the isolated bacterium is a member of the genus *Enterobacter*. Our current isolate suggested that this is the first reported *E.cloacae* that can degrade 2,2-DCP as sole source of carbon. There are very limited studies focused on chlorinated compound degradation by *Enterobacter* species. Other strains isolated from the contaminated soil were identified as *Enterobacter* sp. SA-2, *E. cloacae* D1 and *Enterobacter asburiae* B-14 (Singh *et al.*, 2004; Lacayo-Romero *et al*., 2005; Adebusoye *et al*., 2007).

The whole genomic DNA of *E. cloacae* (accession number: CP001918) was sequenced by Ren *et al.*, (2010). Cof-like hydrolase of *E. cloacae* subsp. *cloacae* ATCC 13047 was identified in the gene. Since MN1 has basic properties of *E. cloacae* therefore, the current putative dehalogenase gene maybe associated with Cof-like hydrolase. In addition, the primers used to screen the dehalogenase gene or to investigate the gene responsible for dehalogenation process were designed based on haloacid dehalogenase like

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                                                                  M   V
1   CGC TGT CTG GGC TTC CGG GGT GAG CAC TGC GGG CTG CGC AAC GCC ATG GTG
     G   E   F   G   L   V   M   I   G   A   V   V   A   R   A   A   A
52  GGG GAG TTT GGG TTG GTC ATG ATT GGG GCG GTG GTG GCG CGC GCC GCG GCG
     K   P   E   K   E   R   T   T   L   G   N   G   Y   E   R   S   A
103 AAG CCG GAA AAA GAA CGA ACG ACC CTG GGC AAC GGG TAT GAG CGT TCA GCT
     D   G   G   R   H   G   W   H   L   P   D   R   R   Q   N   L   Q
154 GAT GGT GGT CGA CAT GGA TGG CAC CTT CCT GAT CGA CGC CAA AAC CTA CAA
     S   R   T   F   S   G   A   A   A   R   M   K   E   Q   G   I   R
205 TCG CGA ACG TTT TCT GGC GCG GCT GCG CGT ATG AAG GAG CAA GGT ATT CGC
     F   V   V   A   S   G   N   R   Y   Y   D   M   I   S   F   F   A
256 TTT GTG GTG GCC AGC GGT AAC CGG TAT TAC GAC ATG ATC TCC TTC TTC GCG
     D   I   D   H   D   V   A   L   V   D   Y   N   G   P   W   L   V
307 GAC ATT GAT CAT GAT GTT GCG TTG GTC GAT TAC AAC GGG CCT TGG CTG GTC
     R   A   G   E   D   V   F   N   S   E   L   S   K   T   H   F   Y
358 CGC GCC GGA GAA GAT GTT TTT AAC TCA GAA CTG TCT AAA ACG CAC TTC TAC
     T   V   A   A   V   L   N   D   V   P   G   I   D   M   I   A   C
409 ACC GTT GCT GCC GTG CTA AAC GAC GTT CCC GGC ATT GAC ATG ATC GCC TGC
     G   K   G   S   A   D   T   L   K   A   Y   D   D   E   F   K   A
450 GGC AAA GGC AGT GCC GAT ACG CTC AAA GCC TAT GAC GAT GAG TTC AAA GCT
     I   A   A   K   Y   Y   H   R   G   E   M   V   S   D   F   D   N
501 ATC GCC GCG AAG TAT TAT CAT CGC GGC GAA ATG GTA AGT GAT TTC GAC AAC
     L   N   D   I   F   F   N   F   G   L   N   V   S   D   D   E   I
552 CTG AAC GAT ATT TTC TTC AAT TTC GGA CTC AAC GTT TCT GAT GAT GAA ATC
     P   R   I   Q   A   M   L   H   E   K   L   N   D   I   M   V   P
603 CCG CGC ATT CAG GCC ATG CTG CAC GAA AAA CTC AAC GAC ATA ATG GTG CCC
     V   T   T   G   H   G   S   I   D   L   I   I   P   G   V   H   K

654 GTC ACG ACC GGC CAT GGA AGT ATC GAT CTG ATT ATC CCC GGC GTG CAT AAA
     A   N   G   L   L   I   L   Q   Q   R   W   G   I   D   D   S   W
705 GCC AAC GGC CTG CTG ATC CTG CAA CAG CGC TGG GGC ATT GAC GAC AGC TGG
     V   V   A   F   G   D   S   G   N   D   V   E   M   L   R   Q   S
756 GTG GTG GCT TTC GGT GAC AGC GGG AAC GAT GTG GAG ATG CTG CGC CAG TCA
     G   F   S   F   A   M   A   N   A   R   P   H   I   K   A   A   A
807 GGC TTC AGC TTT GCG ATG GCG AAT GCC AGA CCG CAT ATT AAA GCA GCG GCC
     R   F   E   A   P   Q   N   N   E   E   G   V   L   D   V   I   D
858 CGC TTT GAA GCA CCG CAA AAT AAC GAG GAA GGC GTA CTG GAT GTG ATT GAT
     K   V   L   N   R   E   A   P   F   N   Stop
909 AAG GTG CTC AAC AGG GAA GCA CCG TTT AAT TGA CAC GTG CGC TCT GGT GCC

960 CTC ACC CTG CCC CTC TCC CAC CGG GAG AGG GAA TAA GAA TT
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Figure 1: Nucleotides and deduced amino acids sequence from *Enterobacter cloacae* MN1. The highlighted area shows HAD-domain of the primary structure. ATG: start codon; TGA: stop codon.

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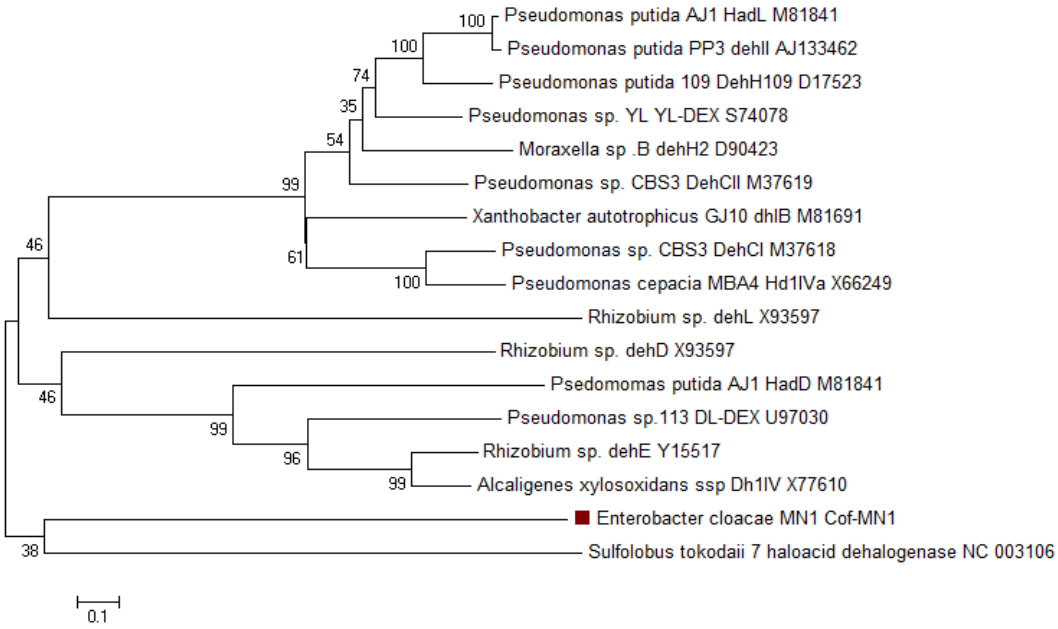

Figure 2: Neighbour-Joining tree showing the relationships of Cof-like amino acids from strain MN1 with L-specific haloacid dehalogenases (Schneider *et al*., 1991; Van Der Ploeg *et al*., 1991; Murdiyatmo *et al*., 1992; Kawasaki *et al*., 1992; Jones *et al*., 1992; Kawasaki *et al*., 1994; Nardi-Dei *et al*., 1994; Cairns *et al.*, 1996; Bachas-Daunert *et al*., 2009), non-specific dehalogenases (Brokamp *et al*., 1996; Stringfellow *et al*., 1997; Nardi-Dei *et al*., 1997) and D-specific dehalogenases (Barth *et al*., 1992; Cairns *et al*., 1996). The name of the proteins and their accession numbers are shown after strain names. The scale bar represents 0.1 substitutions per site.

hydrolases-HAD superfamily (or L-2-haloacid dehalogenase). The PCR product was identified to be a complete Cof-like hydrolase gene associated with L-haloacid dehalogenases. The HAD-domain was also detected in the amino acid sequence. The phylogenetic studies indicated that, there were distance differences between our protein and the rest of haloacids. Therefore, the current finding suggests that the identified protein is equivalent to a new protein able to act on haloacids.

CONCLUSION

In conclusion, using molecular approach could be used in finding a new genus/species and gene of interest of the isolated microorganisms. We have further analyzed strain MN1 and its evolutionary relationship of the gene encoding dehalogenase to Cof-like hydrolase. To the best of our knowledge, this is the first study that demonstrates a novel Cof-like hydrolase associated with dehalogenase from *Enterobacter* that allow this bacterium to grow on halogenated substrate as sole source of carbon. However, its relationship with current dehalogenases and its specialized protein function is far from clear and needs further investigation. In future, enzyme assay and study of mutant strain are necessary to elucidate the protein characterization and its stereospecificity

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