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: halocid dehalogenase (HAD), 2-haloacid dehalogenase, 2-haloacid halohydrinhydrolyase, 2-haloalkanoic acid dehalogenase, 2-haloalkanoic acid halidohydrolyase, 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., 2010a, 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
was grown effectively on selective minimal medium containing 2,2-DCP as a sole source of carbon and energy. Furthermore, we identified a CoI-like hydrolase gene presumably responsible for bacterial growth in 2,2-DCP. The bacterium was subjected to 16S rRNA analysis for genus and species identification.

**MATERIALS AND METHODS**

**Soil sample collection**

Soil samples were collected from the seaside area of Tigaian, 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 K2HPO4·3H2O (42.5 g/L), NaH2PO4·2H2O (10.0 g/L) and (NH4)2SO4 (25.0 g/L). The trace metal salts solution was a 10X concentrate that contained nitriloacetic acid (NTA) (1.0 g/L), MgSO4 (2.0 g/L), FeSO4·7H2O (120.0 mg/L), MnSO4·4H2O (30.0 mg/L), ZnSO4·H2O (30 mg/L) and CoCl2 (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 A600nm and the release of chloride ions at A460nm 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 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 MEGAS 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 Enterobacteriaceae 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   | +                |
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-halocacid 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 tokodai* 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

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

| Closest genus/species names                  | Accession no. | Identity | e-value |
|---------------------------------------------|---------------|----------|---------|
| *Enterobacter cloacae* E717                 | EF059885.1    | 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     |

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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.
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; Kawasaki et al., 1994; Jones et al., 1992; Kawasaki et al., 1994; Nardi-Dei 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.

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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