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ORIGINAL ARTICLE

Characterisation of *Arthrobacter* sp. S1 that can degrade α and β-haloalkanoic acids isolated from contaminated soil

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Abstract A bacterium identified as *Arthrobacter* sp. S1 by 16S rRNA was isolated from contaminated soil. This is the first reported study that *Arthrobacter* could utilize both α-halocarboxylix acid (αHA) [2,2-dichloropropionic acid (2,2-DCP) and D,L-2-chloropropionic acid (D,L-2-CP)] and β-halocarboxylix acid (βHA) [3-chloropropionic acid (3CP)] as sole source of carbon with cell doubling times of 5±0.2, 7±0.1, and 10±0.1 h, respectively. More than 85 % chloride ion released was detected in the growth medium suggesting the substrates used were utilized. To identify the presence of dehalogenase gene in the microorganism, a molecular tool that included the use of oligonucleotide primers specific to microorganisms that can grow in halogenated compounds was adapted. A partial putative dehalogenase gene was determined by direct sequencing of the PCR-amplified genomic DNA of the bacterium. A comparative analysis of the deduced amino acid sequence data revealed that the amino acid sequence has a low identity of less than 15 % to both group I and group II dehalogenases, suggesting that the current putative dehalogenase amino acid sequence was completely distinct from both α-haloacids and β-haloacids dehalogenases. This investigation is useful in studying the microbial populations in order to monitor the presence of specific dehalogenase genes and to provide a better understanding of the microbial populations that are present in soil or in water systems treating halogenated compounds.

Keywords *Arthrobacter* sp. S1 · Dehalogenase · 2,2-dichloropropionic acid · Degradation · Dehalogenase gene

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Introduction

Halogenated organic compounds are produced by chemical synthesis and are found widely throughout the biosphere. Microbial degradation of different halogenated aliphatic and aromatic compounds generally involves enzyme-catalyzed carbon–halogen bond cleavage. The dehalogenation reactions led to the identification of a variety of dehalogenases and dehalogenation mechanisms (Janssen et al. 1994; Fetzner 1998). Nowadays, dehalogenases not only have potential applications in environmental technologies but also in the chemical industry (Kurihara 2011). Originally, dehalogenase enzymes were classified according to their substrates' specificities (Slater et al. 1995). Due to rapid development of molecular techniques and tools, two dehalogenase gene families have been properly assigned to group I and group II deh families (Hill et al. 1999). All the dehalogenase genes assigned to these two families have been isolated from contaminated or polluted environments.

Haloalkanoic acid dehalogenases (HAD) that belong to group I act on C2-halogenated short chain aliphatic acids that include L-isomer-specific and D-isomer-specific. Group I, also known as non-stereo-specific class, catalyze the dehalogenation of D-2-chloropropionic acid (D-2-CP), L-2-chloropropionic acid (L-2-CP) and D,L-2-chloropropionic

acid (D,L-2-CP). In contrast, most of the well-known dehalogenases are L-isomer-specific or group II. Group II only dehalogenate L-2-CP. In the rhizobial system, production of more than one dehalogenases was reported based on substrates' specificities (Cairns et al. 1996; Stringfellow et al. 1997). So far, degradation of αHA by bacteria have been commonly reported in the literature (Kurihara et al. 2000; Kurihara and Esaki 2008). However, degradation of 3CP (3-chloropropionic acid) or βHA that are widely used as intermediates for synthesis of pharmaceutical and pesticides are not well studied, and the mechanism of enzyme action is far from clear (Bollag and Alexander 1971; Jing et al. 2008; Mesri et al. 2009; Lin et al. 2011).

A bacterium isolated from soil by elective culture on 2,2-dichloropropionic acid (2,2-DCP) was identified as an *Arthrobacter* sp. strain S1 by 16S rRNA analysis. The discovery of new dehalogenases from bacteria is still a highlighted area of research (Huang et al. 2011). This paper describes the characteristics of the microbial species isolated from contaminated soil. A better understanding of this microorganism may reveal novel dehalogenase enzyme(s) to stimulate better biodegradation activity in future. The current research, using the PCR technique, aims to identify a putative dehalogenase gene that may be responsible for bacterial growth on 2,2-DCP, D,L-2-CP and 3CP as sole source of carbon and energy.

Materials and methods

Bacteria cultivation and growth experiment

The soil sample was taken from contaminated soil in an area in Bacolod City, Philippines, by Professor R. Gicana from the Microbiology Laboratory College of Arts and Sciences, University of Negros Occidental-Recoletos (UNO-R). All strains were cultivated aerobically at 30 °C on solid minimal media containing 20 mM of 2,2-DCP. Using the streak plate method, the sample was repeatedly streaked on the same type of medium to obtain a pure colony. A pure culture was then subjected to further analysis in liquid minimal media.

The liquid minimal media was prepared as 10× concentrated basal salts containing $K_2HPO_4{\cdot}3H_2O$ (42.5 g/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 10× 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), $MnSO_4{\cdot}4H2O$ (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 10× basal salts and 10 ml of 10× trace metal salts per 100 ml of distilled water, and were autoclaved (121 °C, for 15 min). Carbon sources [2,2-dichloropropionic acid (2,2-DCP), D,L-2-chloropropionic acid (D,L-2-CP), D-2-chloropropionic acid (D-2-CP), L-2-chloropropionic acid (L-2-CP), 3-chloropropionic acid (3CP), monochloroacetate (MCA), dichloroacetate (DCA), and trichloroacetate (TCA)] were sterilized separately and added aseptically to the media to the desired final concentration. The extent of growth was determined by measuring the absorbance at A_{680nm}. Measurement of free halide released during the dehalogenation reaction was carried out by an adaptation of the method of Bergman and Sanik (1957). A sample (1 ml) from the liquid growth medium was added to 100 μl of 0.25 M ferric ammonium sulphate in 9 M nitric acid and thoroughly mixed. To this was added 100 μl mercuric thiocyanate-saturated ethanol and the solution was mixed by vortexing. The color was allowed to develop for 10 min and measured on an A_{460nm} spectrophotometer. Halide concentration was determined by comparison of the absorbance of the test sample against a standard curve of known concentrations of halide.

Amplification of 16S rRNA gene and phylogenetic analysis

Bacterial DNA was extracted from bacterial cultures grown on 20 mM 2,2-DCP minimal media using Wizard® Genomic DNA Purification Kit. The polymerase chain reaction (PCR) was carried out to amplify the target DNA fragments using universal primers, Fd1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rP1 (5′-ACG GTC ATA CCT TGT TAC GAC TT-3′) (Fulton and Cooper 2005). DNA amplification was performed for 30 cycles, and the PCR cycle was set as initial denaturation 94 °C for 5 min, followed by cooling, denaturation 94 °C for 1 min, annealing 55 °C for 1 min, and final extension 72 °C for 10 min. Amplicons were purified using QIAGEN® QIAquick PCR purification kit and sequenced by 1st Base® (Malaysia). The phylogenetic analysis was carried out by comparison with the 16S rRNA gene sequence obtained from the GenBank database (Altschul et al. 1990). Multiple sequence alignment of bacteria from various 16S rRNA gene sequence was constructed using MEGA5 software (Tamura et al. 2007).

Amplification of putative dehalogenase gene using group I and II primers

The PCR primers were described by Fortin et al. (1998) and Hill et al. (1999). The source of the primers were obtained from *Xanthobacter autotrophicus dhlB* 314 5′-TCTGGC GGCAGAAGCAGCTGG- 3′ and *dhlB* 637 5′-CGCGCTTGGCATCGACGCTGATG-3′ (van Der Ploeg et al. 1991) belonging to group II deh genes; $dehI_{for1}$ 5′-ACGYTNSGSGTGCCNTGGGT-3′ and $dehI_{rev2}$ 5′-SGCMAKSRCNYKGWARTCACT-3′ (Hill et al. 1999) belonging to group I deh genes. The PCR conditions were set at an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C, followed by a final extension at 72 °C for 10 min.

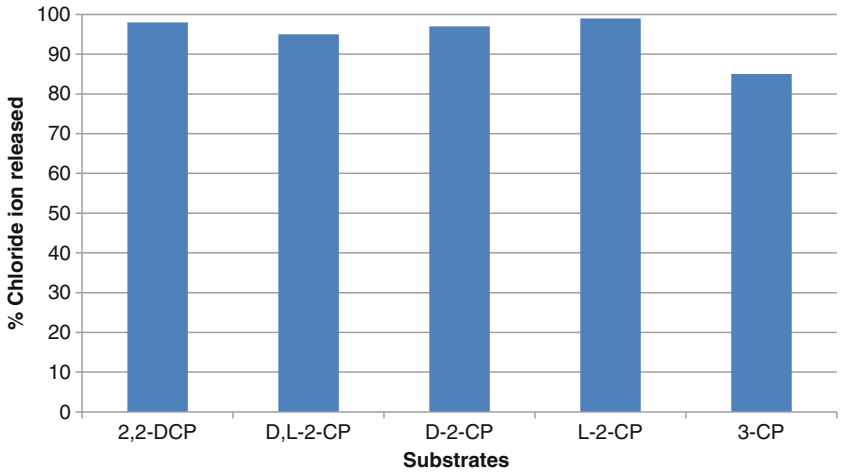

Fig. 1 Percentage of chloride ion released in the growth medium by strain S1. The amount of chloride released in 2,2-DCP, D,L-2CP, D-2-CP, L-2-CP, and 3CP are 98, 95, 97, 99, and 85 %, respectively

The reaction mixture was electrophoresed on a 1 % agarose gel, and purified using QIAGEN PCR purification kit prior sending for sequencing at 1st Base (Malaysia). The DNA sequencing results were converted into amino acid using ExPASy-Translate tool (Gasteiger et al. 2003). The amino acid sequence was analyzed by sequence comparison in the public database using ClustalW2 – Multiple Sequence Alignment programme. Other dehalogenase amino acid sequences were downloaded from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).

Results

Growth experiment of strain S1 on 2,2-DCP and other halogenated compounds

A pure colony of S1 grew well on liquid and solid minimal media supplied with various concentrations of 2,2-DCP up to 20 mM at 30 °C with cell doubling time of 5±0.2 h. However, no growth was detected at 30 mM 2,2-DCP, suggesting that 30 mM of 2,2-DCP is toxic to the cells. A control experiment without 2,2-DCP showed no growth at all. Other growth substrates were tested, such as D,L-2-CP, D-2-CP, L-2-CP and 3CP, which showed growth of up to 20 mM with cell doubling times of 7±0.1, 6.5±0.3, 6±0.2, and 10±0.1 h, respectively. During growth, the chloride ion concentration of the medium was increased up to more than 85 % by the organism (Fig. 1.). Since 2,2-DCP, D,L-2-CP, D-2-CP, L-2-CP and 3CP were the sole carbon sources, the results also indicate that the dehalogenation product were being utilized by the organism for metabolism and growth. Other carbon sources like MCA, DCA and TCA did not show any growth.

The 16S rRNA gene analysis

Bacterium S1 was identified using the 16S rRNA gene sequence analysis. The PCR amplification of 16S rRNA gene was sequenced (1,470 bp) and deposited in NCBI GenBank under accession number KC307960. The highest sequence identity was to *Arthrobacter ureafaciens* showing 94 % identity. Based on this analysis, the bacterium was designated as *Arthrobacter* sp. strain S1. (Fig. 2).

PCR amplification of putative dehalogenase gene and molecular analysis

A PCR fragment of the size of 1.3 kb was generated and the negative control using *E. coli* genomic DNA did not show

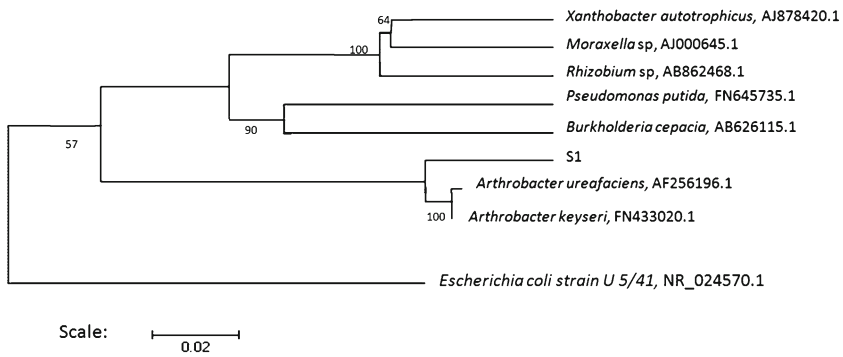

Fig. 2 Neighbor-joining tree based on 16S rRNA gene sequence showing phylogenetic relationships between strain S1 and other related members of the genus. Numbers at nodes indicate level of boostrap support based on a neighbor-joining analysis of 100 sampled datasets. *Scale bar* indicates 0.02 substitution per nucleotide position

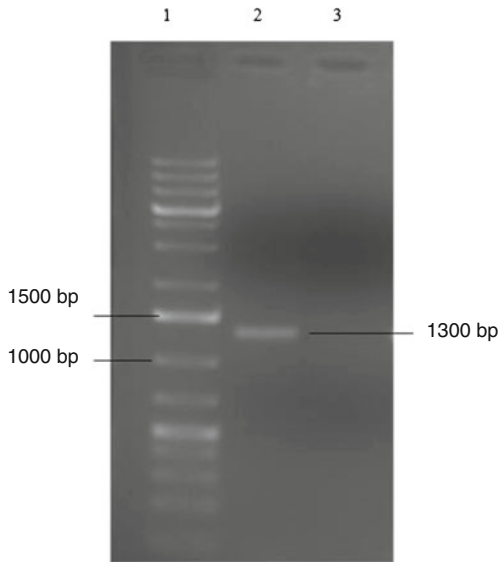

Fig. 3 Agarose gel electrophoresis showing PCR amplified gene fragment of putative dehalogenase gene. *Lane 1* GeneRuler 1-kb Plus Ladder (Fermentas); *lane 2* S1 PCR product of the amplified gene (approximately 1,300 bp); *lane 3* negative control

any amplification (Fig. 3.). In contrast, using degenerate primers described by Hill et al. (1999) did not show any amplification of the same DNA template with *E. coli* genomic DNA as a control.

Analysis of putative dehalogenase DNA and deduced amino acid sequence

The 1.3-kb PCR product was sent for DNA sequencing. The partial gene sequence and the derived amino acid were deposited into NCBI GenBank under accession number KC307961. The partial amino acid sequence was then designated as S1 dehalogenase (DehS1). By obtaining the partial putative dehalogenase gene sequence, its derived amino acid sequence could then be compared with the derived amino acid sequence of dehalogenases in the same group II or group I dehalogenases. Figure 4 shows putative amino acid sequence comparison between S1 and a complete *Xanthobacter autotrophicus* DhlB dehalogenase sequence (van Der Ploeg et al. 1991). The results showed that the identity was only 15 %. Only some of the amino acids are identical to the conserved regions of an important catalytic nucleophile (bold/underline) (Chan et al., 2010). The DehS1 was also compared with a variety of other L-2-haloalkanoic acid dehalogenases (belonging to group II deh), as shown in Fig. 5, such as the HadL from *Pseudomonas putida* AJ1 (Jones et al. 1992); DehH109 dehalogenase from *P. putida* H109 (Kawasaki et al. 1994); and the HdlIV dehalogenase from *Pseudomonas cepacia* MBA4 (Murdiyatmo et al. 1992). However, there were less than 3 % identity with 14 % amino acid similarity.

Strain S1 can grow on 2,2DCP and both D- and L-2CP. Therefore, the amino acid sequence comparison between DehS1 and representative of dehalogenase from group I was also compared (Fig. 6). The three putative key catalytic residues were proposed to be equivalent to DehE and DehI key catalytic residues: Thr64 (Thr62), Glu140 (Glu66), and Asp201 (Asp189) (the equivalent residue of DehE/DehI is indicated in parentheses). These residues were proposed since strain S1 can utilize each of 2,2-DCP, D,L-2-CP, D-2-CP and L-2-CP.

Discussion

Arthrobacter sp. strain S1 was isolated from contaminated soil, using 2,2-DCP as sole carbon source. The bacterial species could also degrade other sources of carbon: D,L-2CP, D-2-CP, L-2-CP, and 3CP. In a previous study, a group of bacteria that can grow on halogenated compounds like αHA were identified but none could grow on βHA (Thasif et al. 2009; Zulkifly et al. 2010). To the best of our

Fig. 4 Amino acid sequence comparison between DehS1 and DhlB. Identical amino acid residues are indicated *asterisks*. *Two dots* indicate amino acid similarity. % identity was 15 % and similarity 10 %. Amino acids with *bold* and *underline* indicate conserved region of an important catalytic nucleophile/catalytic base amino of L-2-haloacid dehalogenases (Chan et al. 2010)

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DehS1      ---------DGVR-RGPPVLPRRRRPAHPRCTAAPRSWTPFQRSSLPAPTSWLPPLLACT
DhlB       MIKAVVFDAYGTLFDVQSVADATERAYPGRGEYITQVWRQK-----QLEYSWLRALMGRY
                     *      *     *    *     : *           ***  *:

DehS1      RTSFALHSA-------------------------------------------SALARQ
DhlB       ADFWGVTREALAYTLGTLGLEPDESFLADMAQAYNRLTPYPDAAQCLAELAPLKRAILSN
             : :                                                  *:  :

DehS1      GAPSTWSAARTAPVSMAVPARSRTPYYAVRLREKQRLRAQYVHPRSPDDYRAFKEVRFAA
DhlB       GAPDMLQALVA-------NAGLTDSFDA-----VISVDAKRVFKPHPDSYALVEEVLGVT
           ***    *  :        *     : *        : *: *    ** *   :**   :

DehS1      RR------TKGLSSMSGAEPGWTARNLASR----VDSYSCSGRNCRLRLHHLPVHACWVV
DhlB       PAEVLFVSSNGFDVGGAKNFGFSVARVARLSQEALARELVSGTIAPLT------------
                  ::*:      : *::   :*      :     **    *

DehS1      HRHWFSPHRRGPGRQLLLPPDAIPFAVPAPTTYAVWGNKFVVVLKVLVYSKLLAGPQDKR
DhlB       ---MFKALRMREE-TY---AEAPDFVVPALGDL----------------PRLVRGMAGAH
               *   *           :*  * ***                    :*: *    :

DehS1      SGVII
DhlB       LAPAV
             .  :
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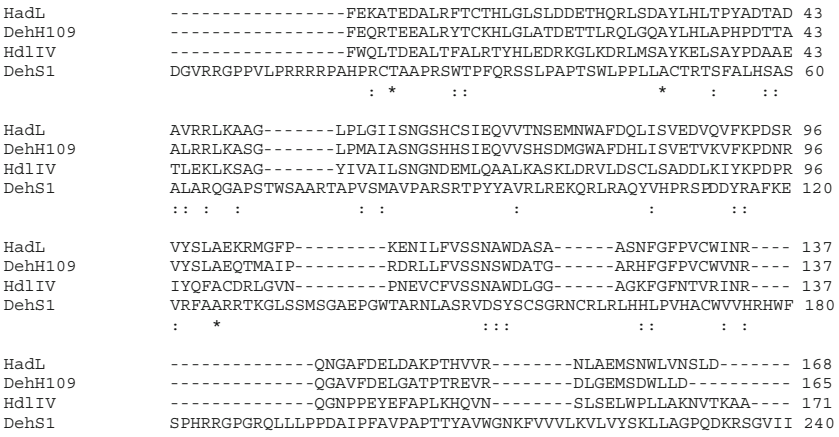

Fig. 5 An overall amino acid sequence comparison between DehS1 and HadL, DehH109, and HdlIV. Identical amino acid residues are indicated by *asterisks*. *Two dots* indicate amino acid similarity

knowledge, this is the first reported *Arthrobacter* sp. that can degrade both αHA (2,2-DCP, D,L-2CP) and βHA (3CP) as sole sources of carbon. Research on αHA has been established, but little on βHA. *Micrococcus denitrificans* (Bollag and Alexander 1971), *Pseudomonas* sp. B6P (Mesri et al. 2009), *Rhodococcus* sp. HJ1 (Jing et al. 2008), and *Bacillus* sp. CGMCC no. 4169 (Lin et al. 2011) have been reported to have the ability to degrade βHA but not αHA. Only *Alcaligenes* sp. was able to grow on both αHA and βHA four-carbon aliphatic acid except 3CP (Staub and Kohler 1989). Current isolates could grow on both αHA and βHA based on a growth experiment measured at A_{680nm} and chloride ion release measured at A_{460nm}. Therefore, strain S1 has a significant value for pollutant degradation in natural ecosystems with less limitation on substrate specificities.

The dehalogenation mechanism catalyzed by αHA were hydrolytic (Kurihara and Esaki 2008). Dehalogenation of 3CP was always associated with the co-enzyme A (Co-A)-dependent β-oxidation pathway or co-A-independent (Staub and Kohler 1989) and oxygenase-type dehalogenase (Yokota et al. 1986). Lin et al. (2011) suggests that neither co-enzymes nor oxygen (Armfield et al. 1995) affect the dehalogenation process of 3CP. This is in contradiction to the enzyme found in *Alcaligenes* sp. (Staub and kohler 1989) which is co-A-dependent and oxygen-dependent (Yokota et al. 1986). However, *Arthrobacter* sp. strain S1 could grow on both αHA and βHA without a co-enzyme present.

Chan et al. (2010) reported that many microbial genomes carry enzyme families containing dehalogenases. However, sequence-based identification of genuine dehalogenases might be problematic because of the low conservation among these dehalogenases enzymes (Figs. 4, 5, 6). Our study indicated that primers generated from group II dehalogenases were able to amplify a putative dehalogenase

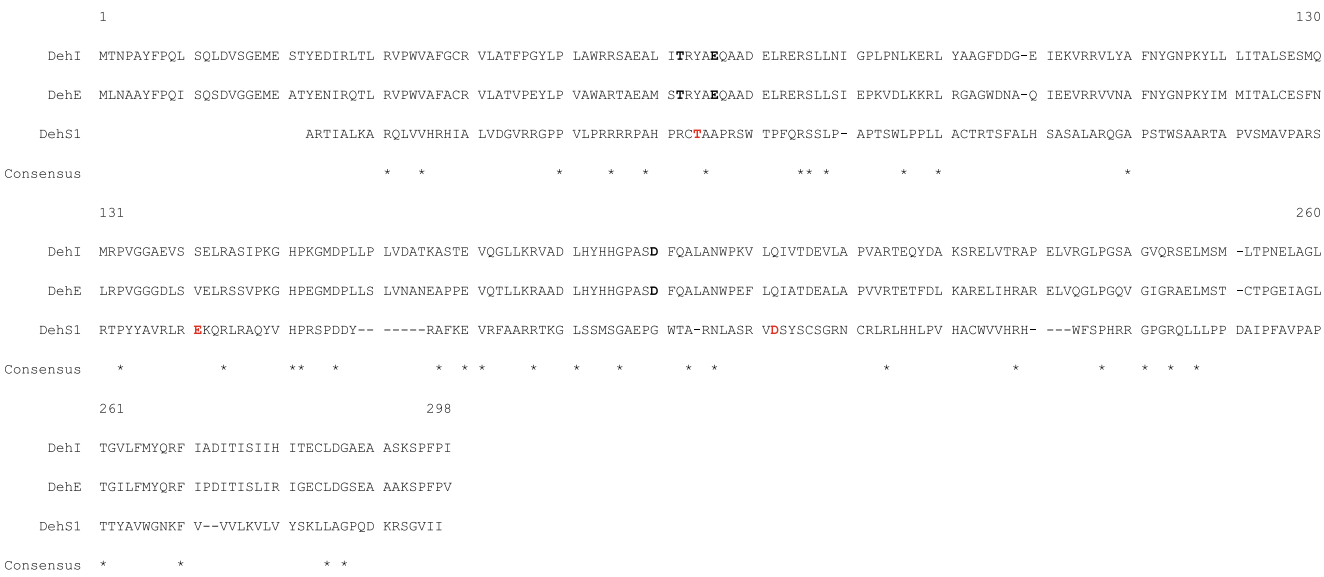

Fig. 6 An overall amino acid sequence comparison between DehI, DehE and DehS1. Proposed identical amino acid residues are indicated by *asterisks*. Proposed catalytic residues of DehS1 marked as *bold/red* are Thr64, Glu140 and Asp201. *Bold* indicates established key catalytic residues of DehE/DehI: Thr62, Glu66 and Asp189 (Kurihara 2011)

gene from *Arthrobacter* sp. S1 that could degrade both αHA and βHA. The sequence has little identity to dehalogenases from either group I or II. Only primers from group II could amplify the putative dehalogenase gene, possibly due to L-2-haloacid dehalogenases being more common than group I dehalogenases. In the current study, the key catalytic residues were proposed as equivalent to DehE and DehI since S1 could utilize 2,2-DCP and D,L-2-CP. In L-Dex, Asp10, Arg41, and Ser118 were involved as key catalytic residues (Kurihara 2011). None of these amino acids matched DehS1 due to only partial DehS1 being obtained. There were two reasons why using group I primers could not amplify the S1 gene: (1) the primers are not universal for some bacteria, and (2) only a single dehalogenase gene may be present that acts on both αHA and βHA. In contrast, *Rhizobium* sp. RC1 was reported to contain more than one dehalogenase (Cairns et al. 1996; Stringfellow et al. 1997). DehE or D,L-haloacid dehalogenase from *Rhizobium* sp. RC1 is unique because it can act on chiral carbons of both enantiomers. It is curious why *Rhizobium* sp. RC1 has more than one dehalogenase, whereby DehE alone can act on all of the substrates that DehD and DehL can also act on. The regulation of dehalogenases is far from clear; however, Huyop and Cooper (2011) proposed a single regulator gene involved in controlling all three dehalogenases. Current investigation suggested that only a single dehalogenase may be present in S1. Its key catalytic residues were proposed to be similar to that of DehE/DehI. This observation was supported by an in silico study of molecular dynamics of docking simulation by Hamid et al. (2012) who suggested that 3CP can bind to the key catalytic residues of DehE. Therefore, the fact that S1 can grow on 3CP may shed a light on key amino acid residues for catalysis. Future work will involve cloning of the full gene to confirm the current analysis.

In conclusion, a molecular approach can be used in screening a new gene of interest. This is the first study that demonstrates that *Arthrobacter* sp. can degrade both αHA and βHA. A putative dehalogenase gene was identified in *Arthrobacter* sp. associated with growth on both αHA and βHA by applying molecular tools using primers belonging to group II dehalogenases. This technique could apply to probing the dehalogenase gene (s) presence in the microorganisms in the soil community or water systems. Only protein characterization studies and site-directed mutagenesis would be necessary to elucidate the enzyme functions, its stereospecificity, and key catalytic residue identification.

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