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# Molecular Identification and Characterization of a Bacterium that has Potential to Degrade Low Concentration of Haloalkanoic Acid

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## ABSTRACT

Most halogenated compounds are major environmental pollutants. Pollution from industrial chemicals and herbicide/pesticide from agricultural activities cause problem to the society. The main objectives were to isolate and characterize the bacterium that can grow in halogenated substrate as the sole source of carbon. A common microbiological and molecular techniques were adapted for further characterization. In current study, a soil microorganism, identified as *Aminobacter* sp. SA1 by partial biochemical and 16S rRNA sequencing method was isolated with 2,2DCP (2,2-dichloropropionic acid) as the sole carbon and energy source. This bacterium has the ability to degrade 2,2DCP (2,2-dichloropropionic acid) and D,L2CP (D,L-2-chloropropionic acid) at 20 mM with cells doubling time of 7 h in 2,2DCP medium. The bacterium was able to grow at low substrate concentration of 1 mM 2,2DCP (20 times lower than normal growth concentration) with cells doubling time of approximately 7 h suggesting the dehalogenase enzyme(s) that present in the bacterium has high affinity towards the substrate. However, at 0.1 mM 2,2DCP (200 times lower than normal concentration) no halide ions were detected in the growth medium suggesting no growth was observed. The investigation highlighted degradation at low substrate concentration suggested that the isolated bacterium can live in low level of halogenated compounds. This observation may or may not involve new kinds of dehalogenases. The presence of more than one dehalogenases in a bacterial system is far from clear.

**Key words:** Halogenated compound, low substrate concentration, 2,2DCP, D,L2CP, oligotroph, dichloropropionate

## INTRODUCTION

Halogenated compounds are widely used as herbicides, insecticides, antibiotics, medicines and so on. These chemicals are produced in large quantities by the chemical industry. In agricultural area, extensive use of herbicides or pesticides led to harmful effects on human and natural environment because any synthetic organohalogens are generally persistent, bioaccumulative and toxic. For this reason widely used of halogenated herbicides received greater attention due to environmental issues (Haggblom and Bossert, 2004).

Many soil microorganisms are capable of utilizing halogen substituted organic acids as their sole carbon sources and the organically bound halogen is liberated as the halide ion

(Fetzner and Lingens, 1994; Schwarze *et al.*, 1997; Olaniran *et al.*, 2001, 2004). The lack of biodegradation is often due to the inability of microorganisms to effectively metabolize compounds with chemical structures to which microorganisms have not been exposed during the course of evolution. In addition, is due to low concentrations of xenobiotic compounds in the environment. However, it is useful to know about growth of microorganisms in low concentrations of pollutants because of the legal requirements. Therefore, if the microorganisms could only remove high concentration of pollutants, they could not be used to meet requirements of the law, since there still will be low concentrations of pollutants in the environment. This study hence considered important since degradation of low concentration of α-chloro substituted haloalkanoic acids such as 2,2DCP and D,L-2-chloropropionic (D,L2CP) are not well studied. Halogenated compounds, 2,2DCP (Dalapon) and D,L2CP are commonly used as herbicides. Among all kind of herbicides, Dalapon and glyphosate were widely used to kill unwanted, sparing non-target type of plants and perennial grasses (Ashton and Crafts, 1973; Hammond and Cooper, 2002).

The isolation and identification of dehalogenase producing bacteria were well studied by Hardman (1991), Leisinger and Bader (1993), Jing and Huyop (2007, 2008), Jing *et al.* (2008), Ismail *et al.* (2008), Thasif *et al.* (2009) and Allison *et al.* (1983). The isolated bacteria possess more than one dehalogenases for example in *Rhizobium* sp. (Huyop and Nemati, 2010). The presence of more than one dehalogenases in one microorganism is far from clear and possibly gives more advantages under fluctuating environment.

A bacterium was isolated previously. This bacterium was capable in degrading 2,2DCP at lower substrate concentration and generates more interest. In this study, we will report the bacterial identification and growth experiment showing bacterial ability to degrade halogenated compound at low substrate concentration.

## MATERIALS AND METHODS

**Growth conditions and media preparation:** The bacterial culture was grown at 30°C using Galenkemp rotary shaker (150 rpm) in 250 mL flasks containing 100 mL minimal salts medium. The liquid minimal media was prepared as 10x concentrated basal salts containing $K_2HPO_43H_2O$ (42.5 g $L^{-1}$), $NaH_2PO_4.2H_2O$ (10.0 g $L^{-1}$) and $(NH_4)_2SO_4$ (25.0 g $L^{-1}$). The trace metal salts solution was a 10x concentrate that contained nitriloacetic acid-$C_6H_9NO_6$ (1.0 g $L^{-1}$), $MgSO_4$ (2.0 g $L^{-1}$), $FeSO_4.7H_2O$ (0.12 g $L^{-1}$), $MnSO_4.4H_2O$ (0.03 g $L^{-1}$), $ZnSO_4.H_2O$ (0.03 g $L^{-1}$) and $CoCl_2$ (0.01 g $L^{-1}$) 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 and were autoclaved (121°C for 15 min at 15 psi). Liquid minimal cultures were supplemented with yeast extract to a final concentration of 0.01% (w/v). Addition of sodium hydroxide (NaOH) will cause the pH increase up to pH 7.5. Oxoid bacteriological agar (15 g $L^{-1}$) is added prior to autoclaving. The carbon source (1 M- 2,2DCP) was neutralized with NaOH (1 M) and sterilized separately by filtration (Acrodisc, with 0.2 μm pore size) and then added aseptically to the autoclaved minimal salts medium to an appropriate final concentration.

**Growth measurement and assay for halide ion:** A 1.0 mL of sample was taken from liquid minimal salts medium aseptically at 2 h interval over 30 h growth period. The growth was measured 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). Sample (1 mL) was added into 100 μL of 0.25 M ammonium ferric sulphate in 9 M nitric acid and mixed thoroughly.

To this was added 100 µL mercuric thiocyanate-saturated ethanol and the solution was mixed by vortexing. The colour was allowed to develop for 10 min and measured at $A_{460nm}$ in a Pye-Unicam SP1750 Series spectrophotometer. Halide concentration was determined by comparison of the absorbance of the test sample against a standard curve of known concentrations of halide.

**16S rRNA gene sequencing:** The genomic DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. PCR was conducted using the prepared genomic DNA as the template. Primers, forward primer, FD1 (5'-aga gtttgatcctggctc ag-3') and reverse primer, rP1 (5'-acg gtcatacctgttacgac tt-3') were used as described by Fulton and Cooper (2005). The PCR product was sent for sequencing at 1st Base Laboratory Malaysia using FD1 and rP1 primers.

**DNA analysis:** The 16S rRNA gene sequencing was carried out by 1st BASE Laboratories Kuala Lumpur. The sequence was further analyzed at http://www.ncbi.nlm.nih.gov/BLAST/, using BLASTn option. The phylogenetic tree was constructed and relatedness by distance matrix was showed using neighbor joining method.

**Reconstruct phylogram using MEGA 4 software:** The bacterial 16S rRNA gene sequence was analyzed as described earlier by Hamid *et al.* (2010). All selected 16S rRNA gene sequences were transformed into FASTA format and analyzed using alignment explorer/Clustal W in MEGA 4. Then, all output data of sequencing alignment by Clustal W were used to reconstruct phylogram. Phylogenetic Neighbor-Joining (NJ) trees were set up by MEGA 4 (Saitou and Nei, 1987). P-distance method was used to compute the evolutionary distances and distances are in the units of the number of base substitution per site (Tamura *et al.*, 2004, 2007). Gaps and missing data were eliminated from dataset.

## RESULTS

**Identification and characterization of *Aminobacter* sp. SA1:** From BLASTn results showed that an unknown bacteria has 96% identity to *Aminobacter* sp. and also matched up to 11 different kinds bacteria (Table 1). The isolated bacterium was then designated as *Aminobacter* sp. SA1.

**Evolutionary relationship of *Aminobacter* sp. SA1 among *Aminobacter* sp.:** The largest genetic distance value was between the sequence of *Aminobacter* sp. SA1 and *Aminobacter* sp. COX (28.722) while the least genetic distance was between *Aminobacter* sp. SA1 and *Aminobacter* sp. BA135 (3.689) as shown in Fig. 1. All closest species obtained were subjected to MEGA 4 to construct the phylogenetic tree. The results showed that *Aminobacter* sp. SA1 was diverged from a same node with *Aminobacter* sp. BA135 and *Aminobacter* aganoensis TH-3. It means there were sister group which had minimum genetic distance and very minimum amount of genetic distance values (Fig. 2).

**Bacteria morphology, staining and identification:** The bacteria colony was observed as a smooth surface, a raised elevation. It formed white colonies on nutrient agar and creamy on 2,2DCP minimal salts medium. It was Gram-negative rod. The partial biochemical characteristics were matched to the genus *Aminobacter* sp. as indicated in Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994). The summary of biochemical tests was in Table 2.

**Cultivation of strain SA1:** From a wide range of compounds tested, 2,2DCP and D,L2CP were the only haloalkanoic acid able to support growth of the organism as the sole carbon and

Table 1: The BLASTn results of the 16S rRNA gene (NCBI database)

| Accession | Description | Max score | Total score | Query coverage (%) | Maxident. (%) |
|---|---|---|---|---|---|
| GU06S311.1 | Bacterium '090512-Mighty 150' | 845 | 845 | 100 | 96 |
| AM1285009.1 | Aminobacter aminovorans A27 | 845 | 845 | 100 | 96 |
| AJ622941.1 | Aminobacter sp. C4 | 845 | 845 | 100 | 96 |
| AF107 722.1 | Aminobacter lissarensis CC495 | 845 | 845 | 100 | 96 |
| NR 02530 1.1 | Aminobacter aminovorans D5M7048 | 845 | 845 | 100 | 96 |
| AB480343 .1 | Aminobacter sp. N91 | 839 | 839 | 100 | 96 |
| EU431701.1 | Uncultured bacterium clone F00S7B_47 | 839 | 839 | 100 | 96 |
| EU748914.1 | Aminobacter sp. BA135 | 839 | 839 | 100 | 96 |
| DQ401867.1 | Aminobacter sp. M5H1 | 839 | 839 | 100 | 96 |
| AJ011 762.1 | Chelatobacter heintzii strain D5M6450T | 839 | 839 | 100 | 96 |
| NR 025302 .1 | Aminobacter niigataensis D5M7050 | 839 | 839 | 100 | 96 |
| NR 028876.1 | Aminobacter aganoensis TH-3 | 839 | 839 | 100 | 96 |
| AF3 7786 7.1 | Uncultured bacterium clone COX1 | 833 | 833 | 99 | 96 |
| AY30 7924.1 | Aminobacter sp. COX | 833 | 833 | 99 | 96 |
| AF246220 .1 | Aminobacter ciceronei C147 | 833 | 833 | 100 | 96 |

[1] #*Aminobacter*_sp._SA1
[2] #*Aminobacter*_aminovorans_A27
[3] #*Aminobacter*_sp._C4
[4] #*Aminobacter*_lissarensis_CC495
[5] #*Aminobacter*_aminovorans_DSM7048
[6] #*Aminobacter*_sp._N91
[7] #*Aminobacter*_sp._BA135
[8] #*Aminobacter*_sp._MSH1
[9] #*Aminobacter*_niigataensis_DSM7050
[10] #*Aminobacter*_aganoensis_TH-3
[11] #*Aminobacter*_sp._COX
[12] #*Aminobacter*_ciceronei_C147

| [1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| [1] | | | | | | | | | | | |
| [2] | 24.686 | | | | | | | | | | |
| [3] | 6.186 | 8.698 | | | | | | | | | |
| [4] | 6.034 | 8.698 | 0.002 | | | | | | | | |
| [5] | 26.317 | 6.005 | 8.369 | 19.763 | | | | | | | |
| [6] | 5.803 | 8.616 | 0.002 | 0.004 | 19.757 | | | | | | |
| [7] | 3.689 | 5.476 | 26.900 | 27.029 | 26.625 | 26.905 | | | | | |
| [8] | 23.129 | 24.166 | 28.525 | 28.525 | 5.449 | 28.572 | 8.843 | | | | |
| [9] | 26.500 | 5.973 | 6.674 | 7.063 | 0.002 | 7.144 | 26.461 | 5.232 | | | |
| [10] | 6.399 | 25.522 | 24.702 | 24.702 | 6.888 | 24.073 | 3.575 | 26.247 | 6.544 | | |
| [11] | 28.722 | 26.905 | 25.180 | 25.180 | 28.522 | 25.529 | 26.033 | 4.872 | 28.527 | 5.103 | |
| [12] | 28.228 | 5.509 | 4.624 | 4.624 | 28.520 | 4.726 | 24.122 | 28.797 | 28.525 | 5.333 | 0.753 |

Fig. 1: The number of base substitutions per site from analysis between sequences. Analyses were conducted using the p-distance method in MEGA4. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 500 positions in the final dataset

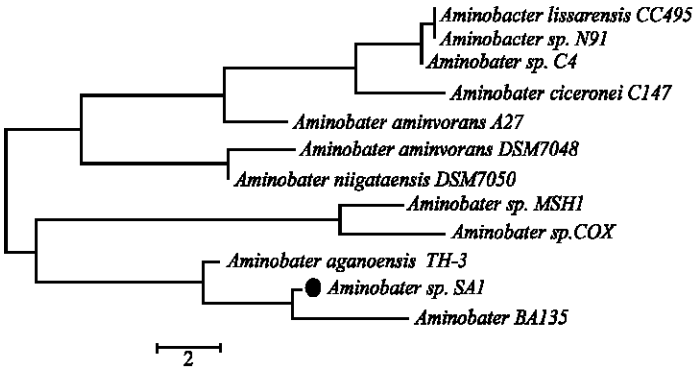


Fig. 2: Phylogenetic tree showing evolutionary relationships of *Aminobacter* sp. SA1 among related species. The scale bar represents 2 substitutions per site

Table 2: Morphology and partial biochemical test

| Test | Results |
|---|---|
| Size | Small (2.0 cm) |
| Shape | Gram (-) Motile, rod |
| Pigment production | - |
| Spore formation | - |
| Motility | + |
| Oxygen requirement | Aerobes |
| Growth at 10°C | - |
| Growth at 30°C | + |
| Growth at 45°C | - |
| Oxidase | + |
| Catalase | + |
| Gelatin hydrolysis | - |

+: Accepted, -: Rejected

energy source. The following halogenated compounds did not support growth of the isolate: 3-Chloropropionic acid (3CP), Monochloroacetate (MCA), Dichloroacetate (DCA) and Trichloroacete (TCA). This study was important as it can be compared to that of others well documented α-chloro substituted alkanoates.

Cell doubling times were approximately 7 h in shake flask culture at 30°C with 20 mM 2,2DCP but growth was inhibited at 40 mM 2,2DCP. The organism also showed growth at 1 mM (20 times lower than the normal concentration) and liberated free chloride ion was detected in the growth medium as a consequence of the dehalogenation reaction. Growth continued until all available 2,2DCP had been dechlorinated as determined by halide ion assays. However, no chloride ion was detected in the growth medium when cells grown in 0.1 mM 2,2DCP suggesting no growth occurs in the minimal medium.

**Halide ion assay analysis of 1 mM 2,2DCP concentration in the bacterial growth medium:** Bacterial growth medium was analysed to monitor the depletion of 2,2DCP and/or generation of the end-product from the dehalogenation reaction. Growth medium inoculated with *Aminobacter* sp. SA1 showed a large drop in the amount of 2,2DCP (%) indicating that the 2,2DCP was utilised (Table 3).

Table 3: The bacterial culture (*Aminobacter* sp. SA1) was grown in minimal salts medium supplemented with 1 mM 2,2DCP as the sole source of carbon. The chloride ion released and 2,2DCP (%) was assessed by halide ion assay in the growth medium

| Time (h) | Chloride ion released (mM)±SD[a] | % (2,2DCP ± SD[a]) |
|---|---|---|
| 0 | 0.0±0.00 | 99.3±0.57 |
| 6 | 0.5±0.5 | 74.3±0.57 |
| 12 | 1.0±1.0 | 50.0±1.00 |
| 18 | 1.5±0.57 | 25.3±0.57 |
| 24 | 1.9±0.12 | 10.3±0.57 |
| 30 | 2.6±0.58 2 | 10.4±0.50 |
| 36 | 2.3±0.57 | 0.6±0.58 |

[a]: Values are mean of triplicate cultures

## DISCUSSION

*Aminobacter* sp. from different species was nearly similar to *Aminobacter* sp. SA1 (96% similarity using 16S rRNA BLASTn analysis). *Aminobacter* species were isolated from diverse terrestrial and marine environments. Some of these bacteria were known as methyl halide-degrading bacteria which capable of degradation of methyl-halide compound. They potentially play an important role in mitigating ozone depletion resulting from methyl chloride and methyl bromide emissions (McDonald *et al.*, 2002).

BLASTn analysis revealed that *Aminobacter* sp. SA1 16S rRNA shared 96% identities to the various sequences of the *Aminobacter* sp. (Table 1). This result supported partial biochemical tests suggesting genus *Aminobacter* (Table 2).

There were many reported cases associated with *Aminobacter* genus that had highly potential to degrade herbicides/pesticides (Duddleston *et al.*, 2002; McDonald *et al.*, 2005; Muller *et al.*, 2011; Warner *et al.*, 2005). In addition to that, *Aminobacter* spp. was isolated from Dichlobenil-treated soil could degrade and mineralized nanomolar concentrations of the herbicide Dichlobenil and its persistent metabolite 2,6-Dichlorobenzamide (Sorensen *et al.*, 2007). Current analysis agreed with previous results, that, this kind of genus has the ability to degrade haloalkanoic acids and also growth at low substrate concentration and therefore, this microorganism deserves more studies, since there is no reported *Aminobacter* strain that can degrade haloalkanoic acid (2,2DCP).

*Aminobacter* SA1 could grow at 1 mM 2,2DCP and possibly dehalogenase of this kind has low Michaelis-menten-Km values or different dehalogenase(s) might be present in the bacterial system. The low Km values for 2,2DCP of dehalogenase E (0.19 mM) type from *Rhizobium* sp. RC1 possibly suited to dealing with low concentrations of growth substrate and no other enzyme would appear to be necessary (Huyop *et al.*, 2004).

## CONCLUSION

This study provides the identity of dehalogenase producing bacteria. The evolutionary relationship of *Aminobacter* sp. SA1 has been derived from MEGA 4 based on the molecular phylogenetic tree. Both partial biochemicals and molecular identification agreed to each other in determining the genus name of the bacterium. *Aminobacter* sp. SA1 has great potential in degrading substrate at low substrate concentration. Biodegradation of low substrate concentrations of xenobiotic has been neglected field of research.

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