RESEARCH LETTER

Isolation and characterization of haloacetic acid-degrading *Afipia* spp. from drinking water

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Abstract

Haloacetic acids are a class of disinfection byproducts formed during the chlorination and chloramination of drinking water that have been linked to several human health risks. In this study, we isolated numerous strains of haloacetic acid-degrading *Afipia* spp. from tap water, the wall of a water distribution pipe, and a granular activated carbon filter treating prechlorinated water. These *Afipia* spp. harbored two phylogenetically distinct groups of α-halocarboxylic acid dehalogenase genes that clustered with genes previously detected only by cultivation-independent methods or were novel and did not conclusively cluster with the previously defined phylogenetic subdivisions of these genes. Four of these *Afipia* spp. simultaneously harbored both the known classes of α-halocarboxylic acid dehalogenase genes (*deh*I and *deh*II), which is potentially of importance because these bacteria were also capable of biodegrading the greatest number of different haloacetic acids. Our results suggest that *Afipia* spp. have a beneficial role in suppressing the concentrations of haloacetic acids in tap water, which contrasts the historical (albeit erroneous) association of *Afipia* sp. (specifically *Afipia felis*) as the causative agent of cat scratch disease.

Introduction

Haloacetic acids are the second-most prominent class of disinfection byproducts formed during the chlorination and chloramination of drinking water (Krasner *et al.*, 1989). These small organic compounds contain one to three halogen atoms, of which monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), and trichloroacetic acid (TCAA) can account for as much as 95% of the total haloacetic acids formed. The consumption of haloacetic acids has been linked to human health risks, such as cancers and reproductive and developmental effects (Herren-Freund *et al.*, 1987; Swan *et al.*, 1998).

Haloacetic acids are biodegradable under aerobic conditions; their biodegradation has been observed in numerous environments, including soil (Olaniran *et al.*, 2001), wastewater (McRae *et al.*, 2004), and water treatment filters (Zhou & Xie, 2002). There is also a substantial body of literature concerning the identity and physiology of haloacetic acid-degrading organisms. The critical step in the biodegradation pathway is the initial hydrolysis–oxidation reaction, which is catalyzed by an α-halocarboxylic acid dehalogenase (Slater *et al.*, 1997), after which the products are processed through central metabolic pathways (Janssen *et al.*, 2001). These α-halocarboxylic acid dehalogenase enzymes are encoded by two evolutionarily distinct classes of genes (*deh*I and *deh*II) (Hill *et al.*, 1999).

In spite of their importance to public health and their prominence in drinking water, we have a relatively poor understanding of the factors that affect the biodegradation of haloacetic acids in drinking water treatment and in drinking water distribution systems. In fact, haloacetic acid depletion in some drinking water distribution systems has been observed and attributed to biodegradation (LeBel *et al.*, 1997; Williams *et al.*, 1997), while haloacetic acid concentrations have remained stable in other drinking water distribution systems (Singer *et al.*, 1995). The lack of degradation observed in many drinking water distribution systems is likely due to a relatively high concentration of residual

disinfectant that inhibits bacterial activity, but could also be due to a lack of HAA-degrading organisms or other environmental factors (e.g. pH, temperature, and micronutrient limitation). More information on haloacetic acid-degrading organisms from water systems is needed in order to optimize the biodegradation of these compounds in water treatment systems and predict their fate in water distribution systems.

The goal of the present study was to isolate and to characterize haloacetic acid-degrading bacteria from various drinking water systems. From a practical perspective, this research would conclusively demonstrate that haloacetic acid-degrading bacteria are present in some drinking water distribution systems further indicating that biodegradation could be a pertinent loss mechanism. We further hypothesized that the haloacetic acid-degrading bacteria from drinking water systems would be novel, stemming from their survival in very nutrient-poor conditions while in the presence of low concentrations of residual chlorine.

Materials and methods

Bacterial enrichment and isolation

Haloacetic acid-degrading bacteria were successfully enriched from four different water samples. One sample was collected aseptically from a granular activated carbon (GAC) filter that was fed prechlorinated water at a water treatment plant in Pennsylvania. This GAC filter was biologically active, effectively biodegrading haloacetic acids to trace levels ($HAA_5 < 5\,\mu g\,L^{-1}$). A second sample was collected from a 6-in. diameter × 1.5-ft cast iron pipe section that was removed from a water distribution main in St Paul, MN, in June 2006. To prevent contamination from the surrounding soil, the exterior of the pipe was cleaned with a 10% bleach solution before cutting. Also, the interior surface of the wall of the pipe was gently rinsed with sterile, nanopure water before scraping off the biofilm and surrounding corrosion products. The final two samples were collected aseptically by filling autoclaved plastic bottles from a flame-sterilized tap within the Gifford Pinchot State Park water system (Lewisberry, PA) and from Cranfield, UK, respectively. These two samples were shipped to the University of Minnesota on ice and processed within 48 h of collection.

Haloacetic acid-degrading bacteria were first enriched in broth culture before isolation on agar plates. The basal medium for the enrichment cultures contained (L^{-1}): 0.03 g $MgSO_4$, 1.96 g $Na_2HPO_4 \cdot 7H_2O$, 0.37 g KH_2PO_4, 0.50 g NH_4Cl, 0.0006 g $CaCl_2$, and 0.1 mL of SL7 trace mineral solution (Biebl & Pfennig, 1981). After autoclaving and cooling, the basal medium was aseptically supplemented with 1 mM of MCAA, DCAA, or TCAA as the sole carbon and energy source.

Enrichment cultures were inoculated in different ways depending on the type of inoculum. GAC-inoculated enrichment cultures were inoculated with biomass that was detached from 20 g of GAC as described previously (Camper *et al.*, 1985). The pipe wall-inoculated cultures were inoculated with scrapings aseptically collected from at least 10 cm from the cut ends to minimize the risk of sample contamination. The wet pipe wall solids were then ground with an autoclaved mortar and then inoculated into the growth medium. Tap water-inoculated enrichment cultures were inoculated by filtering 1 L of tap water through a nylon membrane (47-mm diameter, 0.2-μm pore size, Millipore Corp.) and then placing that filter in 100 mL of growth medium. Turbid growth was observed in enrichments fed MCAA and DCAA within 6 days; TCAA-fed enrichments exhibited turbid growth within 20 days.

Relatively dense cultures of bacteria were obtained by monitoring the enrichment cultures for MCAA, DCAA, or TCAA and then respiking 1 mM of the parent compound after it was depleted. Enrichment cultures were incubated at room temperature in the dark and shaken at 80 r.p.m. The pH of the enrichment cultures was maintained at 7.2 by the addition of 2 M NaOH, as necessary.

Bacteria were isolated from the enrichment cultures once the parent compound had been depleted four consecutive times. Enrichment culture fluid was directly plated onto washed agar (Krieg & Gerhardt, 1994) suspended in the basal medium recipe (described above) modified to include 25 mM phosphate buffer as well as 5 mg L^{-1} of bromocresol purple (pH indicator) and 10 mM of MCAA, DCAA, or TCAA. Agar plates were incubated at room temperature (21–23 °C) until colonies were visibly detectable (< 3 days). Colonies causing a color change from purple to yellow, due to the release of HCl during haloacetic acid degradation, were putatively identified as haloacetic acid degraders. Such colonies were then streaked onto fresh plates up to three times for further purification. Haloacetic acid-degrading ability was confirmed by incubating each isolate in basal medium spiked with 1 mM of a specific haloacetic acid and observing a decrease in concentration over time.

Biodegradation experiments

Bacteria were grown overnight on 1 mM of parent compound, as described above for the enrichment cultures. Cells were then washed three times and re-suspended in basal medium. A cell suspension of 0.1 mL was then inoculated into 300-mL glass bottles containing 100 mL basal medium simultaneously spiked with as many as seven different haloacetic acids [MCAA, monobromoacetic acid (MBAA), monoiodoacetic acid (MIAA), DCAA, TCAA, dibromoacetic acid (DBAA), or tribromoacetic acid] at a concentration of 35 μg L^{-1}. Control bottles without inoculation were also

prepared to monitor for abiotic losses. All bottles were prepared in duplicate and incubated at room temperature and shaken at 110 r.p.m. Aqueous samples (20 mL) were removed from each bottle, placed in a 40 mL glass vial containing 0.2 mL of 2.5% $HgCl_2$ solution to halt biodegradation. Experiments were identified as 'positive' for biodegradation if more than 85% of the original concentration of haloacetic acid was depleted while a killed control exhibited negligible depletion.

Analytical chemistry

For routine analysis of enrichment cultures, haloacetic acids were monitored using a capillary electrophoresis analyzer (Zhang *et al.*, 2004). During the biodegradation experiments, the haloacetic acid concentrations were quantified following EPA method 552.3 (US EPA, 2003) using a Hewlett Packard 6890 gas chromatograph equipped with an electron capture detector (GC/ECD) and a Rtx-1701 capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness).

Genomic DNA extraction

Bacterial isolates were either grown in basal medium supplemented with 1 mM of a haloacetic acid or grown on R_2A agar plates (if growth was especially slow in liquid media). Cells grown in liquid culture were centrifuged and resuspended in 0.5 mL of lysis buffer (5% sodium dodecyl sulfate, 120 mM sodium phosphate); cells grown on R_2A agar were aseptically scraped from the agar and then placed in 0.5 mL of lysis buffer. Cells in lysis buffer were then subjected to three freeze–thaw cycles and a 90-min incubation at 70 °C. Genomic DNA was then purified using a Fast DNA Spin Kit (MP Biomedicals LLC, Irvine, CA).

PCR

PCR was used to amplify 16S rRNA gene fragments from the bacterial isolates using primer set 338F and 907R as described previously (Ghosh & LaPara, 2007). PCR was also used to amplify *deh*I and *deh*II gene fragments from all isolates as described previously (Hill *et al.*, 1999). A second PCR was also used for haloacetic acid-degrading bacteria that did not apparently harbor either *deh*I or a *deh*II gene. This PCR used primers targeting the DL-DEX Mb gene from *Methylobacterium* sp. CPA1 (Omi *et al.*, 2007).

PCR was also used to generate fingerprints of bacterial genomes to help identify clonal strains (Dombek *et al.*, 2000). Briefly, PCR using the BOX-AR1 primer was performed for 30 cycles consisting of 92 °C for 30 s, 50 °C for 1 min, and 65 °C for 8 min. Gel images were normalized and statistically analyzed using BIONUMERICS version 1.5 software (Applied Maths, Kortrijk, Belgium).

Membrane hybridization

Southern analysis was performed using a DIG high prime DNA labeling and detection kit, as per the manufacturer's instructions (Roche, Chicago, IL). Approximately, 1000 ng of DNA was digested with PstI, run on a 0.8% agarose gel, and transferred to a positively charged nylon membrane. Positive controls included the plasmid DNA from PAW6 (*deh*I) and the genomic DNA from *Afipia felis* strain D4 (*deh*II), which we isolated from wastewater (data not shown). The probes were made using gel-purified *deh* PCR products from PAW6 (*deh*I) or D4 (*deh*II). The probes contained a total of 31.25 ng of DNA per membrane reaction. Hybridizations were performed overnight under low stringency conditions ($T = 42$ °C).

Nucleotide sequence analysis

Following PCR, amplicons were purified using a GeneClean kit (MP Biomedicals LLC). Nucleotide sequence analysis was performed at the Biomedical Genomics Center at the University of Minnesota using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the original PCR primers as sequencing primers. All reported sequences are the consensus of bidirectional sequence information. Reported nucleotide sequences do not include the original PCR primer sequences. Nucleotide sequences have been deposited in the GenBank database under accession numbers FJ417095–FJ417116.

Data analysis

Phylogenetic trees were constructed using DNAMAN version 4.1 software. Amino acid sequences were inferred from the nucleotide sequences and then optimally aligned against the inferred amino acid sequences from reference sequences obtained from the GenBank database. Dendrograms were then constructed using the neighbor-joining method (Saito & Nei, 1987).

A polyphasic approach was used to identify clonal strains capable of biodegrading haloacetic acids. Strains were initially distinguished from each other based on the sequence of their 16S rRNA gene fragments. Strains with identical 16S rRNA gene sequences were subsequently distinguished by the nucleotide sequence of their *deh*I and/or *deh*II genes and by their BOX-PCR genomic fingerprint.

Results and discussion

Mixed bacterial cultures capable of growing on three haloacetic acids (MCAA, DCAA, and TCAA) were initially enriched at relatively low substrate concentrations ($\leq$ 1 mM). Using this approach, we were able to successfully enrich bacteria from a GAC filter (Hershey, PA), two different tap waters (Lewisberry, PA; Cranfield, UK), and

one water distribution system pipe wall (St Paul, MN). We were unsuccessful in enriching for haloacetic acid-degrading bacteria from three other tap waters (St Paul, MN; Newport News, VA; Everett, WA) (data not shown).

From these enrichment cultures, we isolated numerous bacteria based on their ability to grow on MCAA, DCAA, or TCAA (Table 1). Especially prominent among these strains were six different *Afipia* spp., which were isolated on each of the three different haloacetic acids and from three different locations from which samples were collected (note: *Afipia* spp. with identical 16S rRNA gene sequences had different BOX-PCR patterns; data not shown). Four of the *Afipia* spp. harbored both *deh*I and *deh*II genes (strains GD1, GTs, EMD1, and EMD2), and *Afipia* sp. strain P1M1 harbored only a *deh*II gene. In contrast, *Afipia* sp. strain GD2 apparently harbored neither a *deh*I nor a *deh*II gene as suggested by negative PCR results (both sets of PCR primers) and negative membrane hybridization/probing results targeting both *deh*I and *deh*II genes (data not shown).

The phylogenies of the α-halocarboxylic acid dehalogenase genes within the *Afipia* spp. were characterized by determining the nucleotide sequences of the PCR-amplified *deh*I and *dehII* gene fragments (Figs 1 and 2). The inferred amino acid sequences from the *deh*I genes from strain GTs clustered within subdivision B of the known *deh*I genes, which consisted of sequences from previously isolated bacteria (Marchesi & Weightman, 2003) (Fig. 1). In contrast, the other three genes (from strains GD1, EMD1, and EMD2) clustered more closely to each other than to any known subdivision (Fig. 1), suggesting a novel gene cluster. Similarly, the *deh*II genes from these *Afipia* spp. clustered either within subdivision C (strains P1M1 and GTs), which consists exclusively of sequences obtained without cultivating individual bacterial strains (Marchesi & Weightman, 2003), or these genes did not cluster with any known subdivision (Fig. 2).

In addition to the *Afipia* spp., four other bacterial strains were also isolated from these enrichment cultures (Table 1). Three of these isolates (strains GM1, GM2, and GM3) were from the MCAA-degrading culture enriched from a GAC filter. All three of these isolates contained a *deh*II gene that

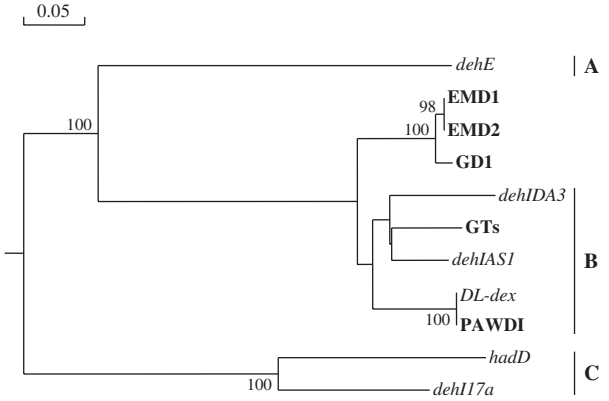

Fig. 1. Dendrogram showing the phylogenetic relatedness of inferred amino acid sequences of the four dehalogenase *deh*I genes (shown in bold) detected in this study. Reference sequences are shown in italics [GenBank accession numbers: *dehE* (Y15517), *DL-dex* (AB301951), *dehIAS1* (AJ511295), *dehIDA3* (AJ430683), *hadD* (M81841), *dehI17a* (AJ133457)]. Bootstrap values are shown for nodes with > 50% probability of 500 replicates. The scale bar indicates an estimated change of 5%.

Table 1. Description of haloacetic-acid degrading bacteria enriched and isolated from a granular activated carbon filter in Hershey, PA (PA-GAC Filter), a tap water in Lewisberry, PA (PA-Tap Water), a tap water in Cranfield, UK (UK-Tap Water), and a water distribution pipe wall in St Paul, MN (MN-Pipe Wall)

Inoculation source/substrate	Strains	*deh*I	*deh*II	MCAA	MBAA	MIAA	DCAA	DBAA	TCAA	TBAA
PA-GAC Filter										
MCAA	*Burkholderia* sp. strain GM1	–	+	+	+	+	–	ND	–	ND
MCAA	*Herminiimonas* sp. strain GM2	–	+	+	+	+	+	ND	–	ND
MCAA	*Burkholderia* sp. strain GM3	–	+	+	+	+	–	ND	–	ND
DCAA	*Afipia* sp. strain GD1	+	+	+	+	+	+	+	+	+
DCAA	*Afipia* sp. strain GD2	–	–	+	+	+	+	ND	–	ND
TCAA	*Afipia* sp. strain GTs	+	+	+	+	+	+	+	+	+
PA-Tap Water										
DCAA	*Methylobacterium* sp. strain PAWDI	+	–	+	+	+	+	–	–	+
UK-Tap Water										
DCAA	*Afipia* sp. strain EMD1	+	+	+	+	+	+	+	+	+
DCAA	*Afipia* sp. strain EMD2	+	+	+	+	+	+	+	+	+
MN-Pipe Wall										
MCAA	*Afipia* sp. strain P1MI	–	+	+	+	+	–	–	–	–

Isolates are described with respect to their ability to degrade multiple haloacetic acids as well as the presence/absence of *deh*I and *deh*II genes.
ND, not determined.

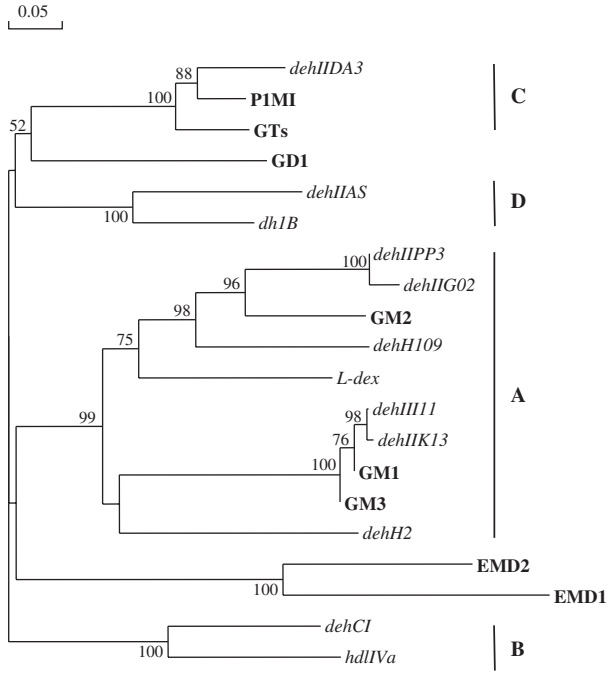

Fig. 2. Dendrogram showing the phylogenetic relatedness of inferred amino acid sequences of the dehalogenase *deh*II genes detected among bacteria isolated from drinking water (shown in bold) during this study. Reference sequences are shown in italics [GenBank accession numbers: *dehIII11* (AJ133465), *dehIIK13* (AJ133466), *dehH2* (D90423), *dehIIPP3* (AJ133462), *dehIIG02* (AJ133464), *dehH109* (D17523), *L-dex* (S74078), *dehIIAS1* (AJ511323), *dh1B* (M81691), *dehIIDA3* (AJ133463), *dehCI* (M62908), *hdlIVa* (X66249)].

phylogenetically clustered with subgroup A of the previously described *deh*II genes (Fig. 2), which are exclusively from bacteria previously isolated and grown in laboratory culture (Marchesi & Weightman, 2003). The remaining bacterial isolate (strain PAWDI) was from a DCAA-degrading culture enriched from tap water in Lewisberry, PA. This bacterium is a *Methlyobacterium* sp., which is curious because this genus is most well known for growing on C1 substrates (Hanson & Hanson, 1996). Furthermore, strain PAWDI did not contain either a *deh*I or *deh*II gene amplifiable by the PCR primers described by Hill *et al.* (1999). Strain PAWDI, however, did contain a *deh*I gene amplifiable by an alternative PCR primer set, which was identical to a previously described *deh*I gene from a *Methylobacterium* sp. (Omi *et al.*, 2007).

We also characterized the ability of all of the bacterial isolates to biodegrade as many as seven different haloacetic acids (Table 1). All 10 bacterial isolates were able to biodegrade all three monohalogenated acetic acids (MCAA, MBAA, and MIAA). In contrast, the majority of the bacterial isolates were unable to degrade TCAA, although the *Afipia* spp. containing both a *deh*I and a *deh*II gene were capable of degrading all seven haloacetic acids. *Afipia* sp. strain GD2, which apparently contained neither a *deh*I gene nor a *deh*II gene, was also capable of degrading DCAA, but not DBAA or any of the trihalogenated acetic acids.

Our research demonstrates that bacteria capable of biodegrading haloacetic acids exist within drinking water distribution systems. Our results, therefore, support the interpretation of previous researchers who have concluded that biodegradation was responsible for reductions in the concentrations of haloacetic acids within drinking water distribution systems. From a practical perspective, our results suggest that microbial activity within drinking water distribution systems could help suppress the concentrations of haloacetic acids, which could have substantial benefits for improved public health due to the potential adverse health effects of haloacetic acids.

Our research also demonstrates that haloacetic acid-degrading *Afipia* spp. commonly occur within drinking water treatment and distribution systems. This result is further supported by our recent study in which *Afipia*-like *deh*II genes were directly detected in four different tap waters (Leach *et al.*, 2009). Both of these results suggest a novel and beneficial role for *Afipia* spp., which have been historically, though erroneously, identified as the causative agent of cat scratch disease (Jerris & Regnery, 1996).

In conclusion, our research demonstrates that *Afipia* spp. capable of biodegrading haloacetic acids can exist within drinking water distribution systems and GAC filters treating prechlorinated tap water. These bacteria are likely important in suppressing the concentrations of these compounds in drinking water, which would help protect public health. Furthermore, the *Afipia* spp. appear to be unique with respect to their genes that encode for α-halocarboxylic acid dehalogenases.

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