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ORIGINAL ARTICLE

In silico characterization of a novel dehalogenase (DehHX) from the halophile *Pseudomonas halophila* HX isolated from Tuz Gölü Lake, Turkey: insights into a hypersaline-adapted dehalogenase

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Abstract Halogenated compounds represent potential long-term threats to human well-being and health and, therefore, the quest for microorganisms capable of degrading these hazardous substances merits urgent consideration. We have isolated a novel dehalogenase-producing bacterium from the hypersaline environment of Tuz Gölü Lake, Turkey and subsequently identified this isolate as *Pseudomonas halophila* HX. Under optimal culture conditions (pH 8.0, 15% NaCl, 30 °C, 200 rpm, 96 h culture time), the strain almost completely degraded (99.3%) 2,2-dichloropropionic acid (20 mM). The dehalogenase gene (*dehHX*) of the bacterium was amplified by PCR, and the deduced amino acid sequence of the DehHX was found to belong to a Group I dehalogenase and to share an 82% sequence identity to the dehalogenase DehI of *Pseudomonas putida* strain PP3. Interestingly, the pI of DehHX was more acidic (pI 3.89) than those of the non-halophilic dehalogenases (average measured pI 5.95). Homology-based structural modeling revealed that the surface of DehHX was unusually negatively charged due to the higher presence of acidic residues, which accounts for the uncommonly low pI seen in DehHX and explains the mechanism of adaptation that contributes to the exceptional halotolerance of the enzyme. The excess surface acidic residues were beneficial in enhancing the water-binding capacity, a crucial feature for preserving the stability and solubility of DehHX in highly saline conditions. In summary, we suggest that bio-prospecting for halogenated compound-degrading microorganisms in highly saline environments is a practical and safe strategy for the bioremediation of contaminated coastal areas.

Keywords Biodegradation · 2,2-Dichloropropionic acid · *Pseudomonas halophila* · Halostable dehalogenase

Introduction

Halogenated compounds liberated by natural and/or industrial processes generally contain high concentrations of four halides, i.e., fluoride (F^-), chloride (Cl^-), bromide (Br^-), and iodide (I^-), all of which are well-known environmental pollutants (Slater et al. 1995). While there are more than 5000 types of naturally occurring halogenated hydrocarbons (Gribble 2009), the ever increasing number of manufacturing activities and cases of illegal dumping of halogenated compounds into water bodies (Oren et al. 1992) further increase their presence in the environment. An estimated 5% of the highly halogenated industrial effluents released into the environment enter saline or hypersaline water systems (Lefebvre et al. 2012), with 2,2-dichloropropionic acid (2,2-DCP) (also known as Dalapon), a highly toxic and recalcitrant biocide, being one of the commonly found contaminants (Häggblom et al. 2000; Van Pée and Unversucht 2003). Most worrying is the persistence of 2,2-DCP in the environment, suggesting that this compound has the potential to pose long-term risks to both

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environmental and human health (Birnbaum and Fenton 2003; Hayes et al. 2006; Qing Li et al. 2006). 2,2-DCP that originates from man-made effluents has been discovered in food sources, especially those caught in marine environments, such as fishes and prawns that constitute a substantial proportion of the human diet (Duarte et al. 2009). An added factor to consider is that the natural bioaccumulation process further concentrates such pollutants in marine food sources, making the situation even worse (Besseling et al. 2013; Chua et al. 2014). Concerted efforts that focus on microbial prospecting in search of dehalogenase-producing bacteria to "clean up" halogen-polluted water bodies (Wang et al. 2011) and marine environments are therefore urgently needed.

The aim of this study was to bio-prospect in hypersaline habitats harboring unique and ancient halophilic microbes capable of surviving or thriving in halogen-contaminated marine or hypersaline environments. Halophiles are salt-loving microorganisms that thrive under extreme salt conditions (up to 35% NaCl) (Castillo-Carvajal et al. 2014). Such exceptional microorganisms have been found in several locations throughout the world, such as the Dead Sea, Israel (Wei et al. 2015), Lake Urmia, Iran (Mehrshad et al. 2015), the solar salterns, Tunisia (Baati et al. 2010), the Tuzkoy salt mine, Turkey (Birbir et al. 2004), the hypersaline environments in southern Spain (Sanchez-Porro et al. 2003), the Dagong Brine Well, China (Xiang et al. 2008), and the Great Salt Lake, USA. In terms of salt tolerance, these microorganisms can be categorized into five major classes based on the concentration of NaCl required for their growth: (1) non-halophiles, < 0.2 M (approx. 1%) NaCl; (2) mild halophiles, 0.2–0.5 M (approx. 1–3%) NaCl; (3) moderate halophiles, 0.5–2.5 M (approx. 3–15%) NaCl; (4) borderline extreme halophiles, 1.5–4.0 M (approx. 9–23%) NaCl; (5) extreme halophiles, 2.5–5.2 M (approx. 15–32%) NaCl (Kushner and Kamekura 1988). Several species of dehalogenase-producing bacteria have been isolated from a variety of halogen-contaminated marine environments (Chiba et al. 2009; Novak et al. 2014; Zhang et al. 2014; Meng et al. 2015). Aquatic environments are among the most important long-term reservoirs of highly halogenated organic pollutants (Zanaroli et al. 2015; Matturro et al. 2016). Consequently, such sites are also excellent environments for isolating halogen-degrading microorganisms, suggesting that the application of these well-adapted microorganisms for cleaning up halogen-contaminated marine or hypersaline environments (Oren 2010) may prove possible.

According to literature, the rate and extent of microbial-assisted degradation of pollutants are profoundly influenced by several geochemical and physical parameters, including temperature (Mohn and Stewart 2000; Eriksson et al. 2001; Haritash and Kaushik 2009), salinity (Kästner et al. 1998; Díaz et al. 2002; Badejo et al. 2013), nutrients (Dibble and Bartha 1979; Chen et al. 2008; Tejeda-Agredano et al. 2010), and the availability of electron acceptors, such as oxygen (Tang et al. 2006; Uribe-Jongbloed and Bishop 2007; Haritash and Kaushik 2009; Ortega-Calvo and Gschwend 2010). Likewise, the distribution and abundance of a given type of extreme environment can also influence biota specialization and microbial evolution (Ve Habitatlar 2002). Since little is known about dehalogenase-producing halophiles and given their innate stability in highly saline environments, the characterization of their bioremediation capacities and a better understanding of the mechanism(s) employed in this process are essential. Although several other microbial dehalogenation mechanisms have been identified, including thiolytic, oxidative, and reductive mechanisms, hydrolytic dehalogenation is the most common mechanism described (Kurihara and Esaki 2008; Fetzner 2010). To date, isolation studies focusing on enzyme-producing bacteria from the hypersaline Tuz Gölü Lake (Turkey) have focused on microbes producing enzymes such as amylases, cellulases, caseinases, gelatinases, lipases, catalases, and oxidases (Birbir et al. 2007), with the exception of dehalogenases. Several strains of *Pseudomonas* sp. capable of degrading hydrocarbons and halogenated compounds (Chandra et al. 2013) have been successfully isolated, but none were found to be halotolerant. To the best of our knowledge, this is the first report detailing a dehalogenase-producing halotolerant bacterium from Tuz Gölü Lake. We describe an aerobic and halotolerant 2,2-DCP-degrading bacterium identified as *Pseudomonas halophila* that produces a dehalogenase called DehHX. For this dehalogenase to competently function under highly saline conditions, we anticipated that special adaptive features would be present in the protein of the halophilic bacterium. Hence, in addition to isolating a halotolerant bacterium we investigated the haloadaptative mechanism within the protein of the *P. halophila* that gives DehHX its halotolerant property.

Materials and methods

Samples and reagents

Water samples were collected aseptically from the shallow shores (average depth 0.2 m) of the halogen-contaminated hypersaline lake Tuz Gölü, near the Van area in Turkey (39° 2′ 50″ N; 33° 26′ 1″ E). The 2,2-DCP was obtained from Sigma-Aldrich (St. Louis, MO), and solutions of 2,2-DCP were filter sterilized through a 0.2-μm nylon membrane disc (Hybond N; Amersham, Little Chalfont, UK) before use. All types of media used in this study were prepared using distilled water. Organic solvents and other chemical reagents were procured from Sigma-Aldrich and were of analytical purity.

Media preparation

The experiment involved the preparation of two different stock solutions. The first stock solution contained basal salts, such as $NaH_2PO_4 \cdot 2H_2O$ (10.0 g l^{-1}), $K_2HPO_4 \cdot 3H_2O$ (42.5 g l^{-1}), and $(NH_4)_2SO_4$ (25.0 g l^{-1}), and the second stock solution contained trace metal salts, such as $MgSO_4$ (2.0 g l^{-1}), $FeSO_4 \cdot 7H_2O$ (120.0 g l^{-1}), $MnSO_4 \cdot 4H_2O$ (30.0 g l^{-1}), $ZnSO_4 \cdot H_2O$ (30 g l^{-1}), $CoCl_2 \cdot 6H_2O$ (10 g l^{-1}), and nitrilotriacetic acid (1.0 g l^{-1}), dissolved in distilled water (Hareland et al. 1975). The growth medium consisted of 2,2-DCP (20 mM) and was autoclaved (121 °C, 15 psi, 15 min) before the addition of various concentrations of filter-sterilized solutions of NaCl (0, 150, 250, and 300 g·l^{-1}). The high salt concentration in the liquid growth medium was to ensure the selective growth of only halotolerant strains capable of utilizing 2,2-DCP as the sole source of carbon and energy. The pH of the growth medium was adjusted to pH 8 ± 0.2, and incubation was at 30 °C with shaking at 200 rpm for 5 days.

A pure colony of the halophilic microorganism was isolated using a minimal medium [1.5% (w/v) Oxoid bacteriological agar No. 1 (Oxoid Ltd., Basingstoke, UK, as the solidifying agent)]. The medium was sterilized [121 °C, 15 psi, 15 min] prior to addition of a filter-sterilized solution of 2,2-DCP (20 mM). A single colony growing on the minimal medium was selected and subcultured onto fresh plates containing 2,2-DCP (20 mM) as the only carbon source and subsequently incubated for 5 days at 30 °C.

Field emission scanning electron microscopy

The morphological characteristics of a single bacterial culture were assessed using field emission scanning electron microscopy. The bacterial cells were incubated for 24 h in a halophilic broth prepared according to Dundas (1977). The bacterial culture was harvested by centrifugation (5000 *g* for 15 min) and washed twice using distilled water. The sample was fixed in 2.5% (w/v) glutaraldehyde for 1 h and then washed once with distilled water prior to the final fixing treatment in 1–4% (w/v) osmium tetroxide for 1 h. The sample was dehydrated by immersion (10 min) in a series of ethanol/water solutions with increasing percentages of ethanol (25, 50, 75, 95, and 100% v/v) (Moran and Coats 2012). The sample was placed onto an aluminum foil disc and dried in a Leica EM CPD300 critical point dryer (Leica Microsystems GmbH, Wetzlar, Germany) prior to coating with platinum and examination under a Hitachi SU5020 FES electron microscope (magnification 11000 ×; accelerating voltage 2000 V) with an SE(L) detector set at a working distance of 16.2 mm.

Biochemical characterization

Assessments of the physiological and biochemical properties of the bacterial strain, namely, Gram staining, motility test, carbohydrate fermentation, and gelatine hydrolysis (Kloos et al. 1974), were done according to the Bergey's Manual of Determinative for Bacteriology (Garrity et al. 2012). After an overnight incubation in a halophilic broth, oxidase and catalase activities were determined by an oxidation reaction carried out using 1% (w/v) tetramethylenediamine (Faller and Schleifer 1981) prepared in a 2.5% (w/v) hydrogen peroxide solution, and the formation of gas and bubbles, respectively, was evaluated (Colwell and Grigorova 1987). Other biochemical tests also included methyl red, indole production, the Voges–Proskauer reaction, urease production, citrate utilization, and nitrate reduction (Colwell and Grigorova 1987).

PCR amplification of 16S rDNA

Genomic DNA extraction was carried out using the Wizard Genomic DNA Extraction kit reagent (Promega, Madison, WI) as per the manufacturer's protocol. Upon purification, the DNA concentration was ascertained and the DNA stored at −20 °C until further use. PCR amplification of the 16S rDNA was carried out in a mixture (50 μl) that contained 0.1 μl of 27F primer (25 μM), 1 μl of 1492R primer (25 μM), 1 μl dNTPs (10 mM total), 4.0 μl $MgCl_2$ (25 mM), 5.0 μl of 10× PCR buffer, 0.5 μl Taq polymerase (Stratagene Ltd., Cambridge, UK), and 0.5 μl of DNA template (104 ng). The PCR reactions were performed in an Eppendorf Master Cycler Nexus Gradient thermal cycler (Hamburg, Germany) using the following cycling program: an initial denaturation of 1 min at 94 °C, followed by 30 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 40 s), and extension (72 °C, 1 min), with a final extension of 10 min at 72 °C. The bacterial 16S rDNA universal primers 27F (5′-AGAGATTGATCCTGGCTCTG-3′) and 1492R, (5′-GGTTTCCTTGTTACGACAT-3′) were synthesized by First Base laboratories Co., Ltd (Selangor, Malaysia). The PCR products were purified and sequenced by First Base Laboratory Co., Ltd., and the taxonomic analysis was conducted using the GenBank BLAST program in the NCBI database (Altschul et al. 1997). The software MEGA version 6 was used for aligning and evaluating the similarity between the 16S rDNA sequences, and the resultant tree topology was evaluated by bootstrap analysis of the neighbor-joining method (Tamura et al. 2013).

Determining optimum growth conditions

The culture conditions (i.e., 2,2-DCP concentration, pH, temperature, agitation rate, medium salinity) that may affect bacterial growth rate were evaluated using minimal media. For

assessing the effect of concentration and pH, we prepared solutions containing different concentrations of 2,2-DCP (20, 30, 40 mM) and solutions at different pH (6.0, 7.0, 8.0, 9.0, 10.0). The pH of each solution was adjusted using HCl (1 M) or NaOH (1 M). The effect of temperature was assessed at 10, 20, 30, 45, and 40 °C, respectively. The effect of shaking rate was assessed at agitation speeds ranging from 150 to 250 rpm, with intervals of 25 rpm. The effect of medium salinity was evaluated in growth media containing varying concentrations of NaCl (0, 150, 250, and 300 g l^{-1}. Bacterial growth was monitored by measuring the turbidity of the growth culture using a Pye-Unicam SP1750 series spectrophotometer (Pye Unicam, Cambridge, UK). Experiments to determine the optimal 2,2-DCP concentration, pH, temperature, agitation rate, and salinity were based on the bacterial growth rate measured over a 5-day incubation period.

2,2-DCP depletion monitored by high-performance liquid chromatography

Bacterial cells cultured under the ascertained optimal conditions were harvested at 24-h intervals and centrifuged (2000 *g* for 10 min) to remove the insoluble particles. The supernatants were collected and subsequently filtered through a 0.2-μm membrane (Toyo Roshi Ltd., Tokyo, Japan). The high-performance liquid chromatography (HPLC) system (Waters Alliance 2690 HPLC Separations Module; Agilent Technologies, Santa Clara, CA) had two components—a capillary Hi-Plex H column (300 × 7.7 mm, 5 μm) and a photodiode array detector. The mobile phase was 0.005 M H_2SO_4. A negative control sample lacking the bacterial inoculation was run in parallel in each assay. Each 20-μl sample was run through the column at a flow rate of 0.6 ml/min and a temperature of 30 °C. A calibration curve was prepared using a series of varying concentrations of 2,2-DCP solutions.

PCR amplification of the halostable dehalogenase gene (*dehHX*)

Amplification of the partial sequence of *dehHX* was done using the dehI-F (5′-ACGCTGCGGGTGCCATGGGT-3′) and dehI-r (5′-TACTTTGGATTGCCATAGTT-3′) primers. The amplified fragment was later sequenced and aligned using MultAlin software (Corpet 1988). Alignment with several other sequences of known Group I dehalogenases maintained by GenBank revealed that the partial sequence of *dehHX* was highly similar to that of *dehI* (accession no. AY138113). Based on the terminal regions of the *dehI* sequence, primers dehHX-F (5′-ATGACCAACCCGTATTTTCC-3′) and dehHX-R (5′-AATCGGTCACTGGCTATCG-3′) were designed using the software Primer 3 (v. 0.4.0) (Untergasser et al. 2012) and the primers then synthesized at First BASE Laboratories (Fig. 1; see also Electronic Supplementary Material 1 for the strategy scheme of amplifying the full sequence of *dehHX*). PCR amplification was carried out in a thermal cycler (Eppendorf Master Cycler Nexus Gradient; Eppendorf)) using the following PCR conditions: an initial denaturation for 5 min at 94 °C, followed by 30 cycles of denaturation (94 °C, 30 s), primer annealing (58 °C, 40 s), and extension (72 °C, 1 min), with a final extension for 5 min at 72 °C. First Base Laboratories sequenced the PCR product.

Amino acid composition and analysis

The frequency of occurrence of each amino acid residue in DehHX and other non-halophilic members of the Group I dehalogenases was calculated using MEGA 6 (Tamura et al. 2013). Outputs were exported into an Excel spreadsheet to calculate the average number of each amino acid, and the results were plotted onto an Excel worksheet. The overall average of hydropathicity value for each protein was calculated using ProtParam (Gasteiger et al. 2005).

Structural analysis of DehHX

The DehHX structure was built using SWISS-MODEL software (Biasini et al. 2014) with DehI as the template. The initial DehHX structure was subjected to structural evaluation using the software packages VERIFY-3D (Liithy et al. 1992), PROCHECK (Laskowski et al. 1993), and ERRAT (Colovos and Yeates 1993), respectively. The model was subjected to molecular dynamics refinement using the parallel version of GROMACS 4.5.1 and the Gromos96 53a6 force field, run on an Ubuntu computer having a 1.6-GHz processor and a quad-core processor (Van Der Spoel et al. 2005). The system volume, temperature (30 K), and number of particles for the ensemble were held constant. The relative electrostatic surface potential of the model was calculated by APBS (Lerner and Carlson 2006) in PyMOL (DeLano 2002).

Results

Characterization of a new bacterium capable of using 2.2-DCP as the sole carbon source

By incubating a water sample from the hypersaline lake Tuz Gölü in minimal medium containing NaCl (25%, w/v) and 20 mM 2,2-DCP, we were able to isolate a new halotolerant bacterium that could use 2,2-DCP as the sole carbon source. After incubating the newly isolated bacterium at 30 °C for 5 days on agar containing 2,2-DCP, we isolated pure colonies (red in color and approx. 2–4 mm in diameter). Interestingly, no colonies were observed in the medium which did not contain 2,2-DCP. The isolates were Gram-negative, rod-shaped

Fig. 1 Amplification strategy to obtain the full sequence of the dehalogenase gene of *Pseudomonas halophila* HX (*dehHX*). The complete *dehI* sequence was obtained from the NCBI database (accession no. AJ133460.1). The *dehHX* coding region is shown in *bold*, and the start and stop codons are *shaded*. Primers used to amplify the fragment and the complete *dehHX* sequence are *underlined and labeled*

microorganisms with an average cell size of 0.7 × 1.9 μm. They grew optimally at pH 7.9 and were extremely halotolerant, being able to grow on media supplemented with NaCl concentrations up to 25% (w/v). The isolates were motile with high catalase and oxidase activities. They digested gelatine, casein, and citrate substrates although they were, unable to utilize mannitol and glucose. Additionally, they were not capable of reducing nitrate to nitrite. Interestingly, their biochemical properties (Table 1) were similar to those previously described for *P. halophila* DSM 3050 (Sorokin et al. 2006).

Identification of the isolated bacterium as a *P. halophila* strain according to its 16S rDNA gene sequence

The 16S rDNA gene sequence (GenBank accession no. KR071871) of the bacterium was obtained as a continuous stretch of 1450 bp, and the sequence was found to be almost identical (99%) to those of other *P. halophila* strains. We constructed a phylogenetic tree using 16S rDNA gene sequences, and the result indicated that the new bacterium is a close relative to *P. halophila* DSM 3050 (GenBank accession no NR-117120.1) (Fig. 2). We named the new isolate *P. halophila* HX.

Optimum growth conditions for *P. halophila* HX

The optimum growth conditions for the *P. halophila* HX isolates were assessed by varying several parameters, including 2,2-DCP concentration (10–30 mM), pH (6.0–9.0), temperature (10–35 °C), and agitation rate (150–250 rpm). The optimum conditions that rendered the highest growth of *P. halophila* HX after 96 h of incubation time were 30 °C, pH 8.0, and an agitation rate of 200 rpm. These optimal conditions were subsequently applied for the culturing of *P. halophila* HX in growth media containing increasing concentrations of NaCl using only 2,2-DCP as the sole carbon and energy source. Figure 3 shows the growth curves for *P. halophila* HX monitored at varying concentrations of NaCl. The bacterium grew well in the highly concentrated media and tolerated NaCl concentrations as high as 25%

Table 1 Comparison of the main morphological and biochemical characteristics for *Pseudomonas halophila* HX and *P. halophila* DSM 3050

Feature	*P. halophila* strain HX	*P. halophila* strain DSM 3050 (Sorokin et al. 2006)
Shape	Fat Rods	Rods
Size (μm)	0.67–0.81 × 1.58–2.22	0.8–1.0 × 1.5–5.0
Pigmentation	Reddish	Reddish-brown
Gram straining	_	_
Motility	+	+
Facultative anaerobe	_	_
Oxidase activity	+	+
Catalase activity	+	ND
NaCl range	0–4.27 M (25% *w/v*)	1.5–4.5 M (26% *w/v*)
Temp range (°C)	10–35	4–37
pH range	7–9	6.7–8.5
Ability to digest starch	_	_
Ability to digest gelatin	+	+
Ability to digest casein	+	+
Ability to digest citrate	+	ND
Nitrate reduction	_	_
Ability to digest lactose	_	ND
Ability to digest mannitol	_	ND
Ability to digest glucose	_	ND
G + C content (mol%)	58	61
Source	Tuz Gölü Lake (Turkey)	Great Salt Lake (Utah, USA)

+, positive reaction; −, negative reaction; ND, not determined

(w/v) (250 g l^{-1}, 4.27 M) and demonstrated a constant cell doubling time of 25.4 ± 0.6 h.

Measuring 2,2-DCP depletion by HPLC

The nearly complete depletion of 2,2-DCP (99.3%) in the growth medium containing 25% (w/v) NaCl as compared to the negative control after a 96-h incubation confirmed that the *P. halophila* HX isolate was capable of utilizing 2,2-DCP as the carbon source. Rapid consumption of the compound occurred mainly within the exponential growth phase (24–72 h). The concentration of residual 2,2-DCP in the growth medium remained relatively stable during the stationary phase (72–96 h), but decreased precipitously during the starvation phase (>96 h; Fig. 4).

Sequencing of dehalogenase gene *dehHX*

To confirm the presence of a dehalogenase gene (*dehHX*) in the isolated *P. halophila* HX, we amplified a fragment of the gene by PCR. The electrophoresed amplified DNA product (503 bp) was similar in size to that identified by Hill and colleagues (1999). Alignment of the *dehHX* fragment sequence with those of other

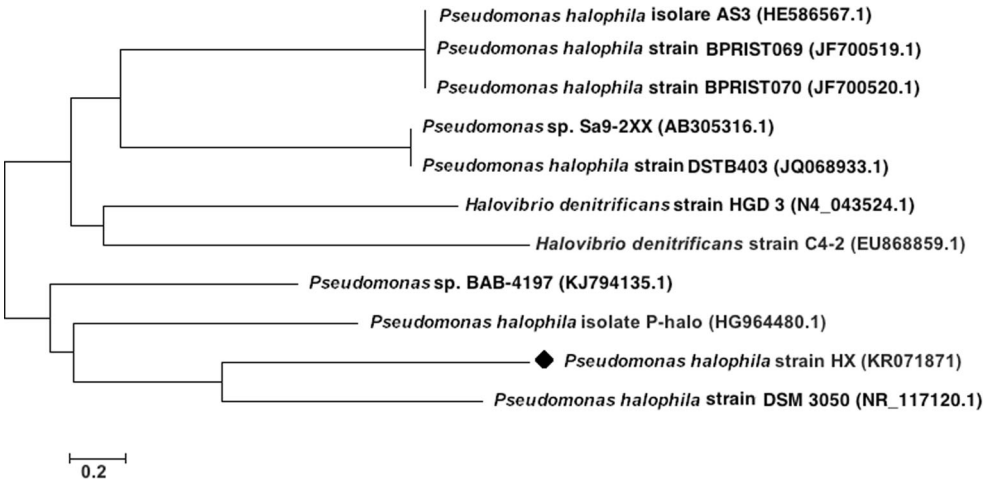

Fig. 2 Neighbor-joining phylogeny tree for the *P. halophila* HX isolate. *Scale bar* represents 0.01% substitution per site. The accession number for each bacterium is obtained from NCBI and presented *in parenthesis*

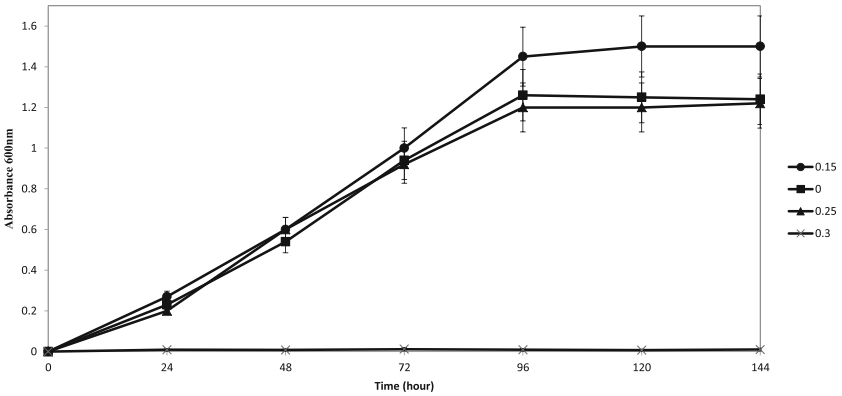

Fig. 3 Growth curves for *P. halophila* HX cultured in medium containing various concentrations of NaCl. *Vertical bars* Standard deviation of the mean

Group I dehalogenases, i.e., *dehI* of *Pseudomonas putida* strain PP3 (Schmidberger et al. 2008), DL-*DEX* of *Pseudomonas* sp. 113 (Nardi-Dei et al. 1997), *dehE* of *Rhizobium* sp. RC1 (Stringfellow et al. 1997), and *HadD* of *Pseudomonas putida* AJ1 (Barth et al. 1992), revealed that *dehHX* has the highest sequence identity (83.1%) with *dehI* (Table 2).

Considering the significant similarity between the *dehHX* fragment and the *dehI* gene, the subsequent step to amplify the complete *dehHX* sequence based on the *dehI* sequence seemed possible. In this study, the complete *dehHX* sequence (approx. 900 bp) was successfully amplified and isolated using the *dehHX* fragment as the template with primers constructed from the *dehI* sequence. The full *dehHX* sequence and its deduced amino acid sequence were deposited in the NCBI GenBank (accession no. KR297065). Pertinently, pairwise alignment of the deduced DehHX amino acid sequence with that of DehI revealed an 82% identity.

Subsequent comparative analysis of the DehHX sequence with those of other Group I dehalogenases revealed that the DehHX amino acid composition was distinctly unlike those of other taxa (Fig. 5) as the DehHX contained a substantially higher number of acidic residues, i.e., 8.27% Asp and 12.06% Glu versus an average of 5.5% Asp and 7% Glu in the amino acid sequences of the non-halophiles. Similarly, the percentages of positively charged residues, i.e., Lys and Arg, were significantly lower in DehHX (1.3 and 2.7%, respectively) than the summed average percentage (12.2%) for dehalogenases from non-halophilic species. Although His can be considered to be a positively charged molecule, the percentage of His in DehHX (1.7%) was found to be comparable with that of the non-halophilic dehalogenases (2.5%). In addition, the overall average of hydropathicity for the DehHX was −0.31, indicating it was more hydrophilic than the non-halophilic homologs, namely, DehI (−0.09), DehE (−0.15), and HadD (−0.19).

Interestingly, the higher number of negatively charged residues found in the DehHX relative to dehalogenases from the non-halophilic bacteria strongly suggests that the surface potential of DehHX is negative, as expected. Correspondingly, the DehHX model structure generated in this study corroborates our hypothesis. The results revealed an unusually large distribution of negatively charged (acidic) residues over the surface of the DehHX protein. The anomaly seen here, therefore, explains the observably overall negative surface electrostatic potential of DehHX (Fig. 6).

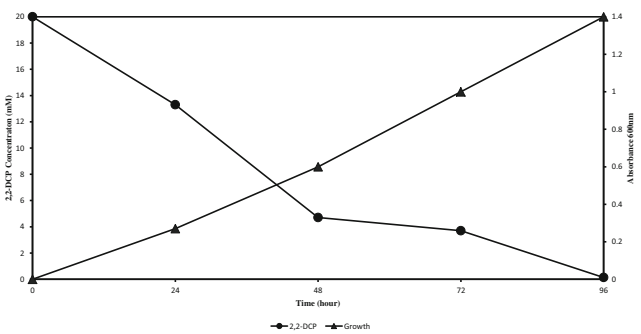

Fig. 4 Relationship between the concentration of 2,2-dichloropropionic acid (*2,2-DCP*) and the exponential and stationary portions of the growth curve of *P. halophila* HX in liquid minimal medium supplemented with 25% (w/v) NaCl and 2,2-DCP (20 mM)

Discussion

The increasing demand for more eco-friendly solutions to clean up saline and hypersaline environments contaminated with halogenated compounds has resulted in the scientific community turning their attention towards the halophilic dehalogenase-producing microorganisms over their non-halophilic counterparts. The rationale behind this choice is that such microorganisms have evolved unique cellular enzymatic machinery that allows them to thrive in extreme saline environments. In our study, we isolated, identified, and characterized a novel halotolerant dehalogenase, DehHX, produced by *P. halophila*. Subsequent in silico assessments of the DehHX protein provided useful insights into the physiological haloadaptation mechanisms which render *P. halophila* HX well-adapted for survival in highly saline environments.

Since halophilic enzymes are defined in terms of the halophily of the organism in which they are found or based on their salt requirements for activity, stability, or solubility (Madern et al. 2000), the results of our study show that the

Table 2 Multiple sequence alignment of a fragment of the dehalogenase gene of *P. halophila* HX (*dehHX*) with other Group I dehalogenase genes

Gene	Identity (%)	Source	Accession number
dehI	83.1	*Pseudomonas putida* strain PP3	AJ133460.1
D,L-dex	52	*Pseudomonas* sp. 113	U97030
dehE	61.3	*Rhizobium* sp. RC1	Y15517
HadD	49.4	*Pseudomonas putida* strain AJ1	M81841

isolated *P. halophila* is well-adapted to using 2,2-DCP as its sole source of carbon, as evident from the almost complete depletion of the compound (99.3%) after 96 h of incubation. These results thus affirm the positive as well as competent utilization of the substance by *P. halophila* HX. Remarkably, the growth of the bacterium was still observable in culture medium containing up to 40 mM of 2,2-DCP; beyond this concentration its viability began to decline, presumably due to the increased toxicity of the compound (Bagherbaigi et al. 2013). Assessment of the effect of salinity on the bacterium revealed that *P. halophila* HX is exceptionally halotolerant, a quality not generally observed in bacteria of the *Pseudomonas* family. These results reveal the *P. halophila* HX was able to retain its viability even at high salt concentrations [25% (w/v) NaCl] and efficiently utilize the 2,2-DCP as its sole carbon source. Based on these characteristics, the utilization of the bacterium as a potentially bioremediation agent for cleaning up hypersaline or marine environments contaminated with halogenated compounds is feasible.

Since the efficacy of *P. halophila* HX to carry out bioremediation processes could be influenced by various factors, optimization of the physical conditions related to microbial-assisted bioremediation processes is necessary (Ventosa 2006). In the case of the effect of pH, our results reveal that the growth of *P. halophila* HX reached the optimum state between pH 6.0 and 9.0, a finding which is in agreement with that of an earlier study (optimum pH ranged from 6.95 to 8.15) on other microbes isolated from Tuz Gölü Lake (Zeki Camur and Mutlu 1996). The preference of *P. halophila* HX for slightly alkaline conditions can be attributed to the naturally high NaCl content in the thalassohaline Tuz Gölü Lake (Litchfield and Gillevet 2002; Oren 2008), although more neutral pH values have also been recorded (Oren 2002). Remarkably, this lake is both naturally alkaline and highly saline because of the seasonal evaporation periods that cause an extremely complex surface accumulation of alkaline soil within the ecosystem (Litchfield and Gillevet 2002; Oren 2008; Steadman and McMahon 2011). In addition to elevating the pH, this phenomenon increases the concentration of NaCl in the lake (Litchfield and Gillevet 2002; Oren 2008), thereby further enhancing the osmotic pressure difference between the aquatic life and the environment. A noteworthy point to highlight here is the ability of DehHX from *P. halophila* HX to efficiently degrade 2,2-DCP under highly alkaline and saline conditions. The efficacy of the bacterium to degrade such toxic compound as 2,2-DCP is unlike those seen in other non-halotolerant dehalogenase-producing bacteria in which maximal catalyzing activities are normally observed at neutral or slightly basic conditions (Abdul Hamid et al. 2015; Liu et al. 2016). Hence, our study results suggest that DehHX has potential value for the self-cleaning and bioremediation of alkaline and saline environments polluted with halogenated hydrocarbons.

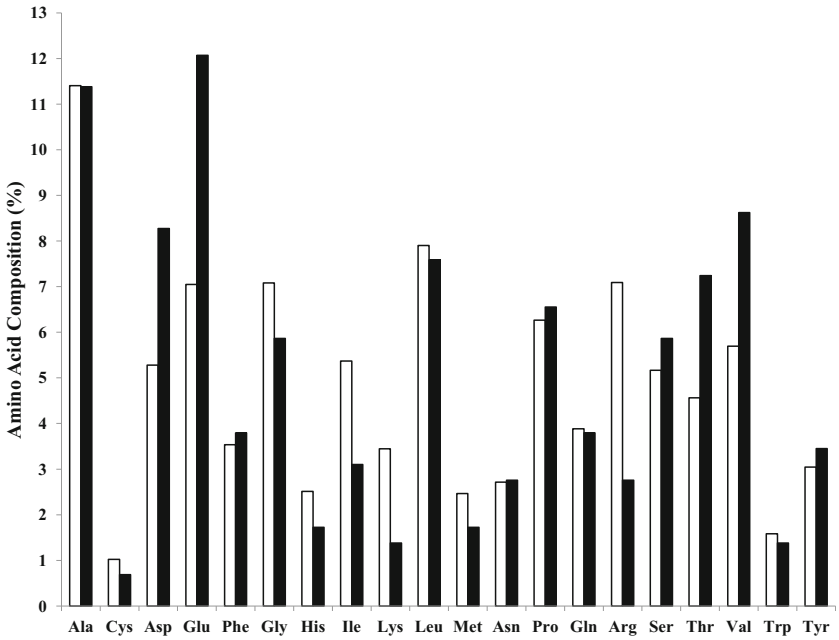

Fig. 5 Bar graph comparing the numbers of each amino acid in the dehalogenase DehHX and the average number of each amino acid in dehalogenases from non-halophilic bacteria. *Black bar* DehHX, *white bar* Group I dehalogenases, including DehE from *Rhizobium* sp., DehI from *Pseudomonas putida* PP3, DL-DEX from *Pseudomonas* sp. 113, and HadD from *Pseudomonas putida* strain AJ1

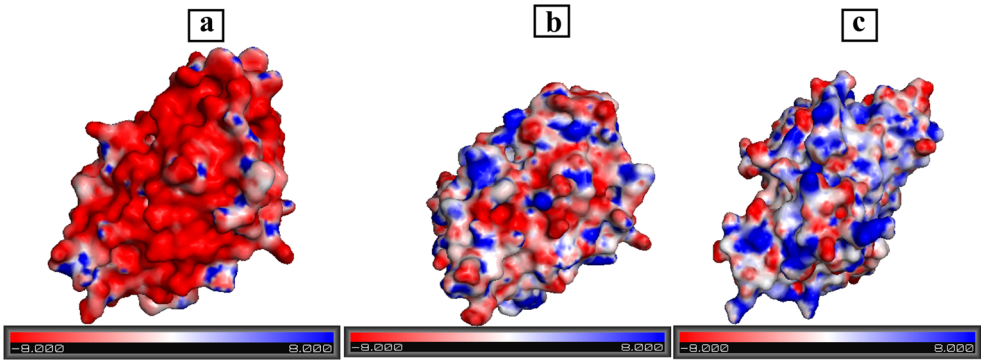

Fig. 6 Surface electrostatic potentials of three dehalogenases. **a** DehHX from *P. halophila* HX, **b** DehI from *P. putida* strain PP3, **c** DL-DEX from *P.* sp. 113. The electrostatic energy is reported in units of kT. *Red* Negative potential, *blue* positive potential

We also examined the effect of temperature because the temperature of a system can influence the equilibrium position of an enzyme–enzyme-catalyzed reaction, as well as the activity and stability of the enzyme (Mohamad et al. 2015). Our results demonstrate that *P. halophila* HX was viable between 10 °C and 35 °C, which is similar to the temperature range reported in an earlier study involving *P. halophila* DSM3050 isolated from the Great Salt Lake in the USA (4–37 °C) (Ventosa et al. 1998). The slight variation in the optimal growth temperatures between these two *P. halophila* strains is possibly due to their adaptation to living in different habitats. In the case of *P. halophila* DSM3050, the temperature range of the Great Salt Lake can vary from 26 °C in mid-July to 4 °C during the spring time (Crosman and Horel 2009), while the temperature fluctuations in the upper regions of the Tuz Gölü Lake water are between 12 °C and 37 °C during the summer/spring (Altın et al. 2012). Hence, it can be inferred that the possible reason for the differences in the optimal pH for these two *P. halophila* strains is the substantial temperature variation between their two habitats.

We also assessed the effect of agitation speed on the growth of *P. halophila* HX and found that agitation speeds between 150 and 250 rpm were appropriate to maintain the necessary amount of dissolved oxygen in the *P. halophile* HX growth medium. This is an important aspect to be considered when cultivating batch cultures of bacteria as a suitable agitation speed would consequently improve bacterial growth by ensuring that the bacterial culture suspension remains properly aerated and the nutrients sufficiently distributed and available. Furthermore, bacterial settlement to the bottom of the flask can result in increased cell death due to the lack of nutrient availability and should be averted in order to result in the production of higher amounts of dehalogenase to catalyze the dehalogenation of 2,2-DCP.

The ability of *P. halophila* HX to tolerate concentrations of NaCl as high as 25% (w/v) (250 g l^{-1}, 4.27 M) with a cell doubling time of 25.4 ± 0.6 h is relatively similar to characteristics reported for *P. halophila* DSM 3050 [up to 26.2% (w/v) 262 g l^{-1}, 4.5 M] (Sorokin et al. 2006). Interestingly, *P. halophila* HX grew well in all culture broths containing NaCl concentrations as high as 25% (w/v). Unlike halophiles, halotolerant microorganisms can grow well in a non-saline environment, as was also observed for *P. halophila* HX. The development of this trait may be due to the heterogeneity of the Tuz Gölü Lake habitat (Sorokin et al. 2006). The salinity of the lake fluctuates regularly in space and time, hence predominantly favoring the survival of euryhaline microorganisms (Sorokin et al. 2006). The increased oligotrophy and high salinity conditions in Tuz Gölü Lake indicate that the non-halophilic dehalogenases would not be able to survive in such environments as highly saline conditions could potentially alter protein folding of their enzymes and disrupt their active conformation. Moreover, changes in the hydrophobic and electrostatic interactions that accompany fluctuations in salinity could also contribute to the structural destabilization and consequent inactivation of enzymes (Sorokin et al. 2006).

Although the average salinity of Tuz Gölü Lake is considerably higher than 35% (w/v) NaCl, we noted that increasing the concentration of NaCl in the growth medium of *P. halophila* to beyond 25% (w/v) adversely affected the bacterium and prevented its multiplication. This outcome may have been due to the lack of carbon-containing nutrients other than 2,2-DCP. An earlier report corroborates our observation, hence signifying the importance of nutrient availability for the survival and propagation of such bacteria in saline environments (Sorokin et al. 2006). However, the relatively broad salinity range preferred by *P. halophila* HX suggests that this bacterium may be a suitable bioremediation agent for many saline environments, such as seawater [3.5% (w/v) NaCl] polluted with halogenated hydrocarbons.

It is wide accepted that a highly saline environment can significantly impact the solubility and stability of a protein and consequently its functionality (Oren 2008). Under such conditions, the protein becomes dehydrated as water becomes less available owing to the phenomenon of water molecules being locked within ionic lattices (Mevarech et al. 2000). This locking phenomenon causes the unfolding of the dehydrated hydrophobic residues located on the surface of the enzyme and subsequent clumping with other protein molecules, resulting in disruption of the protein's stability. To counter such undesirable changes, the negatively charged surface of the DehHX protein, which is unusually rich in acidic residues, may have arisen as an evolutionary adaptive mechanism to allow the acidic residues to interact and bind with the remaining

water molecules in the surrounding saline environment. Additionally, the decreased hydrophobicity in DehHX (related to the high concentrations of surface acidic residues) is another excellent adaptation of the dehalogenase to better compete with ions for water molecules. This adaptative mechanism helps the DehHX dehalogenase to improve its solubilization and hydration (Frolow et al. 1996; Britton et al. 2006; Karan et al. 2012) in such environments. Earlier studies that reported on the crystal structures of halophilic enzymes also demonstrated the presence of exceptionally high numbers of surface acidic amino acids (Frolow et al. 1996; Madern et al. 2000).

Conclusions

The results of this study highlight the isolation of a novel halotolerant bacterium, *P. halophila* HX, from Tuz Gölü Lake which produces a dehalogenase, i.e., DehHX. DehHX from *P. halophila* HX is the first ever Group 1 haloalkanoic dehalogenase isolated from a hypersaline environment. The bacterium was found to almost completely degrade 2,2-DCP after 96 h of incubation (99%) and to utilize this compound as the sole carbon and energy source. Correspondingly, our homology modeling study strongly suggests that *P. halophila* HX has evolutionarily adapted to the extreme hypersaline and alkaline environment of Lake Tuz Gölü, as inferred from the unique DehHX protein generated in this study, which showed the presence of an exceptionally high number of surface acidic residues, in contrary to the non-haloterant dehalogenases. It is worth mentioning here that the unusually high number of surface acidic residues on the DehHX protein suggest that this dehalogenase has a higher water-binding capacity and therefore can remain sufficiently hydrated under highly saline and alkaline conditions. The findings in the in silico study corroborate the efficiency of DehHX to degrade nearly all of the 2,2-DCP, even when this substance is present at very high percentages of NaCl in the growth medium. Correspondingly, our findings confirm our hypothesis that *P. halophila* has adapted well to living under such conditions. In general, we have shown that bio-prospecting for halogen-degrading microorganisms in highly saline environments is not only feasible, but potentially a more practical as well as eco-friendly strategy for cleaning up contaminated coastal areas. The findings reported here further provide useful insights into the engineering and tailoring of specific proteins that could function under extreme industrial and environmental conditions.

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