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Research Article

Desulfoluna spp. form a cosmopolitan group of anaerobic dehalogenating bacteria widely distributed in marine sponges

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One sentence summary: *Desulfoluna* spp. is a cosmopolitan dehalogenating bacterial group distributed in organohalide-containing sponges across different geographic locations.
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Abstract

Host-specific microbial communities thrive within sponge tissues and this association between sponge and associated microbiota may be driven by the organohalogen chemistry of the sponge animal. Several sponge species produce diverse organobromine secondary metabolites (e.g. brominated phenolics, indoles, and pyrroles) that may function as a chemical defense against microbial fouling, infection or predation. In this study, anaerobic cultures prepared from marine sponges were amended with 2,6-dibromophenol as the electron acceptor and short chain organic acids as electron donors. We observed reductive dehalogenation from diverse sponge species collected at disparate temperate and tropical waters suggesting that biogenic organohalides appear to enrich for populations of dehalogenating microorganisms in the sponge animal. Further enrichment by successive transfers with 2,6-dibromophenol as the sole electron acceptor demonstrated the presence of dehalogenating bacteria in over 20 sponge species collected from temperate and tropical ecoregions in the Atlantic and Pacific Oceans and the Mediterranean Sea. The enriched dehalogenating strains were closely related to *Desulfoluna spongiiphila* and *Desulfoluna butyratoxydans,* suggesting a cosmopolitan association between *Desulfoluna* spp. and various marine sponges. *In vivo* reductive dehalogenation in intact sponges was also demonstrated. Organobromide-rich sponges may thus provide a specialized habitat for organohalide-respiring microbes and *D. spongiiphila* and/or its close relatives are responsible for reductive dehalogenation in geographically widely distributed sponge species.

Keywords: anaerobic, debromination, dehalogenation, microbe-host interactions, sponge

Introduction

Sponges (*Porifera*) are filter-feeders with prokaryotic microorganisms as a major component of their diet. While some bacteria are immediately digested, others are retained by the sponge, and it is now well recognized that complex, microbial communities inhabit sponge animals (Hentschel *et al.* 2006, Taylor *et al.* 2007a, Webster *et al.* 2010, 2013, Pita *et al.* 2018). Certain sponges can harbor microbial communities comprising up to 35% of their body mass and at densities of 10^9 cells/g (Hoffman *et al.* 2005, Hentschel *et al.* 2012). These bacteria are enriched in marine sponges over a range of host species and geographical locations (Fieseler *et al.* 2004, Olson and McCarthy 2005, Lafi *et al.* 2009, Olson and Gao 2013). This association between host sponge and associated microbiota may represent an ancient symbiotic or commensal relationship. The presence of this phylogenetically and functionally diverse microbial community gave rise to a classification scheme of high microbial abundance (HMA) vs. low microbial abundance (LMA) sponges (Weisz *et al.* 2008).

An estimated 9000 living sponge species are known, and many produce a vast array of bioactive compounds as secondary metabolites, including diverse halogenated compounds such as brominated phenolics, indoles and pyrroles (e.g. Carté and Faulkner 1981, Norte and Fernandez 1987, Norte *et al.* 1988, Ebel *et al.* 1997, Bowden *et al.* 2000, Utkina *et al.* 2001, Gribble 1999, 2003). *Porifera* date back more than 600 million years to the Precambrian, and have likely produced many of these bioactive compounds during this time, as a potential chemical defense against microbial fouling, infection or predation (e.g. Thoms *et al.* 2006, Haber *et al.* 2011). For example, in *Aplysina aerophoba*, bioactive organobromine compounds can reach more than 10% of the animal dry weight (Teeyapant *et al.* 1993). The organohalogen chemistry of the sponge animal might furthermore select for bacteria that can utilize halogenated compounds as a source of carbon or energy.

Our previous work demonstrated the presence of dehalogenating bacteria within *Aplysina aerophoba* sponges collected from the

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Mediterranean Sea (Ahn *et al.* 2003). Stable anaerobic dehalogenating enrichment cultures using lactate as the carbon source and bromophenols as electron acceptors were maintained and from these cultures we isolated a novel, strict anaerobic bacterium, *Desulfoluna spongiiphila* strain AA1 within the *Desulfobacteraceae* of the phylum *Desulfobacterota* (formerly *Deltaproteobacteria*) that could grow by respiratory reductive debromination (Ahn *et al.* 2009, Liu *et al.* 2017, 2020). A second isolate, *D. spongiiphila* strain DBB was obtained from sediment (Peng *et al.* 2020). Organohalide respiration is mediated by reductive dehalogenases (RDases), encoded by homologous *rdhA* genes (Jugder *et al.* 2015). Three reductive dehalogenase (*rdhA*) genes involved in respiratory metabolism of organohalides were identified within the genomes of *D. spongiiphila* AA1 and DBB (Liu *et al.* 2017, Peng *et al.* 2020). Transcription of one of the three *rdhA* genes was significantly upregulated during respiration of 2,6-dibromophenol and sponge extracts, while transcription of a second *rdhA* gene was upregulated by bromobenzene (Liu *et al.* 2020, Peng *et al.* 2020). In addition, *Desulfoluna* spp. can reduce sulfate. *D. spongiiphila* can thus take advantage of both brominated compounds and sulfate as electron acceptors for respiration.

We postulated that anaerobic organohalogen-respiring bacteria are widespread in different sponge species, forming stable populations with the sponge animal that function in the cycling of sponge-derived organohalide compounds. The objective of this study was to assess whether anaerobic reductive dehalogenating bacteria were associated with sponge species collected from different locations in temperate and tropical waters and whether dehalogenating microbes were active within the sponge animal. Additionally, we wanted to assess if sponge-associated dehalogenating bacteria are cosmopolitan, unique to a geographic site, and/or only associated with a particular sponge species. We also sought to determine whether *D. spongiiphila*, previously isolated from an *Aplysina aerophoba* sponge in the Mediterranean could be detected in other sponge species and locations. For this project, samples from geographically distant and taxonomically distinct sponges were collected (Table 1) to establish enrichment cultures amended with 2,6-dibromophenol (2,6-DBP) as a homologue for the organohalides present in marine sponges. Integrated anaerobic cultivation and molecular analysis-based approaches were used to assess the diversity and occurrence of the dehalogenating bacterial community. Moreover, reductive dehalogenation within live sponge animals was also demonstrated. Our results indicate that *Desulfoluna* spp. is a cosmopolitan anaerobic dehalogenating bacterial group widely distributed and active in different marine sponges.

Materials and methods

Sponge collection and establishment of anaerobic dehalogenating cultures

Sponges were collected from sites corresponding to different marine ecoregions in the Mediterranean Sea, and the Atlantic and Pacific Oceans. Sponges were collected by scuba diving in the Mediterranean Sea at Banyuls Sur Mer, France in May 2005 (42° 28' 59" N and 3° 07' 41" E) and Rovinj, Croatia (45° 05' N, 13° 38' E) in April 2006; in the open sea at Bucco Sur, Tumbes, Peru (03° 56' 43"S, 80 ° 56' 45" W and 03 ° 59' 02" S 80 ° 59' 18" W) in July 2008 and Puerto Rico, Caribbean Sea (18° 27' N and 66° 04' W) in October 2007. Additional specimens were collected in the United States from docks from the shores of the Mullica River-Great Bay Estuary, Tuckerton, New Jersey (39°30'31"N 74°19'33"W) in July 2008; a tidal estuary in Long Island Sound, Connecticut 41° 2' 46" N, 73° 30' 11.98" W) in June 2007. Florida deep-water samples were obtained from Pourtalès Terrace and Florida straits (23° 56'3" N, 80° 55'33"; 24° -81' W) in Summer 2007. In addition, the sponges *Pseudaxinella lunaecharta* (order *Halichondrida*) and *Chondrilla nucula* (order *Chondrosida*) were purchased from LiveAquaria (Rhinelander, WI) for use in *in vivo* assays.

Anaerobic cultures to determine debrominating potential were prepared as described previously (Ahn *et al.* 2003). Briefly, freshly collected sponge material was aseptically homogenized in 0.2 μm filtered seawater, sparged with N_2 to remove dissolved oxygen and 1 to 2 ml of this homogenate inoculated into 30 to 50 ml of anaerobic mineral salts medium (MSM; with or without sulfate) under a headspace of N_2/CO_2 (70:30) in serum flasks capped with rubber stoppers (Ahn *et al.* 2003). The cultures and uninoculated controls were amended from deoxygenated stock solutions with 100–200 μM 2,6-dibromophenol (2,6-DBP; Sigma-Aldrich Chemical Co., Milwaukee, Wis., min. 99% purity) as the electron acceptor and a 1 mM mixture each of lactate, butyrate and propionate, as electron donors. Autoclaved sterile media controls spiked with 2,6-DBP were included with each set of sponge cultures. All cultures were incubated without shaking at 21 to 28 °C in the dark.

Enrichment of anaerobic dehalogenating bacteria from primary sponge cultures

Primary cultures established with different sponges from Croatia, France, Peru, Puerto Rico, and USA that showed dehalogenation activity were maintained and dehalogenating microorganisms further enriched by successive transfers (1/10 dilution) into anaerobic MSM, typically every 1–2 months, and re-spiked with 2,6-DBP (~200 μM) as the electron acceptor and the mixture of short chain fatty acids (typically 1 mM each of lactate, propionate butyrate) as electron donors. Some cultures were maintained with the 20 mM sulfate as an additional electron acceptor. At each transfer uninoculated sterile MSM controls were also prepared and amended the same way. Select enrichment cultures were also tested for dehalogenation of 2,4,6-tribromophenol, 2-iodophenol and 4-iodophenol (100–200 μM; Sigma-Aldrich Chemical Co.).

Isolation of debrominating bacteria was pursued from select enrichments using the anaerobic agar-shake tube technique with 0.8% Noble Agar added to 10 ml MSM containing 1 mM lactate and 200 μM 2-BP in Balch culture tubes. After 4 to 8 weeks of incubation, colonies were harvested using a sterile syringe following strict anaerobic technique. The harvested colonies were transferred to a broth culture containing 100 μM 2-BP and 1mM lactate to test for dehalogenating activity. Broth cultures originating from the agar-shake colonies that exhibited debrominating activity were re-amended with 2-BP and transferred to fresh medium.

At each transfer, DNA was extracted and the bacterial community analyzed by denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (TRFLP) (see Supplementary Material) to determine changes in the microbial community structure and assess whether only a single DGGE band or terminal restriction fragments (TRFs) could be observed (as an indication of a highly purified culture) following previously described methods (Ahn *et al.* 2003, 2007, 2009). If not, the shake agar technique was repeated, single colonies harvested, and inoculated into MSM broth for debromination tests until a pure isolate was obtained. DGGE patterns and 16S rRNA gene TRFLP electropherograms showing common and unique TRFs from enrichment cultures were compared to that of *D. spongiiphila* strain AA1^T (Ahn *et al.* 2009).

Table 1. Debromination activity and presence of *Desulfoluna*-like bacteria in different sponge species collected from coastal sites in the Mediterranean, Caribbean, Atlantic, and Pacific, and deep-water environments in Florida.

Sampling location	Sponge species [a]	Microbial Abundance [c]	Debromination Observed [b]	Detection of *Desulfoluna* 16S rRNA/23S rRNA amplicon
Banyuls sur Mer (France)	*Aplysina aerophoba*	HMA	+	+/+
Rovinj (Croatia)	*Acanthella acuta*	LMA	+	nd
	Aplysina aerophoba	HMA	+	+/+
	Axinella polypoides		-	nd
	Chondrilla nucula	HMA	+	+/+
	Chondrosia reniformis	HMA	+	+/+
	Crambe crambe	LMA	+	-/-
	Dysidea avara	LMA	+	+/+
	Ircinia sp.	HMA[d]	+	-/-
	Mycale sp.	LMA[d]	+	+/+
	Oscarella lobularis		-	nd
	Tethya cf. *limski*	LMA[d]	+	+/+
	Agelas sp.	HMA	-	-/-
Puerto Rico	*Agelas clathrodes* A	HMA	+	+/-
	Agelas clathrodes B	HMA	+	+/-
Long Island Sound (CT, USA)	*Haliclona* sp.	LMA	+	+/+
	Halichondria sp.	LMA[d]	+	+/+
	Microciona sp.		+	+/+
	Clathria prolifera	LMA[d]	+	+/nd
Deep water environments (FL, USA)	*Raspailiidae*	HMA[d]	+	+/-
	Siphonodictyon sp.		-	-/-
	Axinellidae		-	-/-
	Gorgonacea		-	-/-
	Siphonodictyon mucosa		-	-/-
	Leiodermatium sp.	HMA[d]	+	-/-
	Plakortis sp.	HMA[d]	+	-/-
	Desmacellidae		-	-/-
	Spongosorites sp.		-	-/-
Bucco Sur, Tumbes (Peru)	Unidentified (A)		+	nd
	Unidentified (B)		+	nd

[a]Over 20 *A. aerophoba* from Banyuls sur Mer, 9 *A. aerophoba*, 3 *Mycale* sponges and 3 *Tethya* sponges from Rovinj were tested. All other sponge species were single individuals.
[b]Dehalogenation of 2,6-DBP to 2-BP and phenol observed in initial sponge culture within 1–2 months.
[c]Desgination from Maldonado *et al.* (2012) and Gloeckner *et al.* (2014).
[d]some species in the genus/family are known as HMA or LMA
nd, not determined

Analytical methods

Samples of 1 ml were taken aseptically from debrominating cultures and enrichments with N_2 flushed syringes using strict anaerobic technique and filtered with 0.45 μm pore size filters prior to analysis by high performance liquid chromatography (HPLC) to determine the concentrations of 2,6-DBP, 2-bromophenol (2-BP), and phenol over time. An HPLC (Agilent 1100 series or Shimadzu system SIL-10A, SCK-10A, SPD-M10A) with a C18 column (Spherisorb, 4.6 × 250 mm, particle size 5 μm; Phenomenex) was used with methanol/water/acetic acid (50:48:2) as eluent at a flow rate of 1 ml/min, with a Diode Array detector monitoring absorbance at 280 nm. Concentrations were determined by comparisons to 5-point standard curves in the concentration range 100–1000 μM.

Molecular community analysis

Active 2,6-DBP dehalogenating enrichment cultures were sampled for microbial community analysis. Three mL samples were filtered using 10 mm Nucleopore filters (pore size, 0.2 μm; Whatman) and DNA extracted using the UltraClean Soil DNA isolation kit (MO BIO, Solana Beach, Calif.) or a modified phenol-chloroform method (Kerkhof and Ward 1993).

To determine the presence/absence of *Desulfoluna* spp., DNA extracted from debrominating cultures was PCR amplified with *Desulfoluna*-specific primers for the 16S rRNA gene (AA47F: CATGCAAGTCGAACGAGAAA; AA852R: CAATACCCGCGACACCTAGT) and 23S rRNA gene (AA246F: 5′-TCAATAAAGCGCTCAGCGTA-3; AA431R: 3′-TGCAAAATTAATCGCACTGG- 5′). PCR amplification parameters were as follows: 5 min 94°C, 35 amplification cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and a final extension at 72°C for 10 min with conditions as described in Ahn *et al.* (2003). PCR products (expected size ~800 bp) were then separated by electrophoresis on a 1% agarose gel.

Bacterial 16S rRNA gene amplicons for phylogenetic analysis of the various enrichment cultures were amplified as described previously by (Ahn *et al.* 2003). PCR (primer set AA47F-AA852R) amplification parameters were as follows: 94°C 5 min initial melt; 30 amplification cycles of 94°C, 30 s; 55°C 30 s; 72°C 1.5 min and a final extension at 72°C for 7 min. Purified PCR products were sent for Sanger sequencing (Genewiz Inc., North Brunswick, NJ). Phylogenetic trees of the 16S rRNA gene were constructed with MEGA11 (Tamura *et al.* 2021). The evolutionary history was inferred us-

ing the Minimum Evolution method (Rzhetsky and Nei 1992) and evolutionary distances computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004). The Minimum Evolution tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei 1987) was used to generate the initial tree. All positions with missing sequence data were eliminated. The partial 16S rRNA gene sequences have been submitted to GenBank under accession numbers OM864325-OM864339.

In vivo sponge assays

The sponges *Pseudaxinella lunaecharta* (order *Halichondrida*, family *Axinelidae*) and *Chondrilla nucula* (order *Chondrosida*, family *Chondrillidae*) were purchased from LiveAquaria.com. Three individual *Clathria prolifera* (order *Poecilosclerida*, family *Microcionidae*) sponges were collected from a loading dock on the NW shore of Long Island Sound (41° 2' 46.4", -73° 30' 11.98"), stored in zip-lock bags filled with seawater, and transported back to the laboratory. Intact sponges, one *Pseudaxinella lunaecharta* (124.5 g), one *Chondrilla nucula* (not recorded), and three individual *C. prolifera* (6.3 g, 6.7 g, and 6.8 g), were each incubated in tanks with 2 L aged seawater (obtained from the Delaware estuary; 30 parts per thousand salinity and used without any further treatment) aerated with an aquarium air pump and maintained at room temperature (~21 °C). Aerated seawater tanks without a sponge animal served as negative controls. To monitor *in vivo* debrominating activity the tanks were amended with 2,6-DBP to a concentration of ~100–150 μM for *P. lunaecharta* and *C. nucula* and ~600 μM for *C. prolifera*. Samples (1 ml) of the water in the tank were taken over a 2-week period and analyzed for 2,6-DBP and dehalogenation product concentrations by HPLC as described above. Data for *P. lunaecharta* and *C. nucula* are from triplicate water samples analyzed at each time point, while data for *C. prolifera* (3 individual sponges) are for single samples of each sponge incubation.

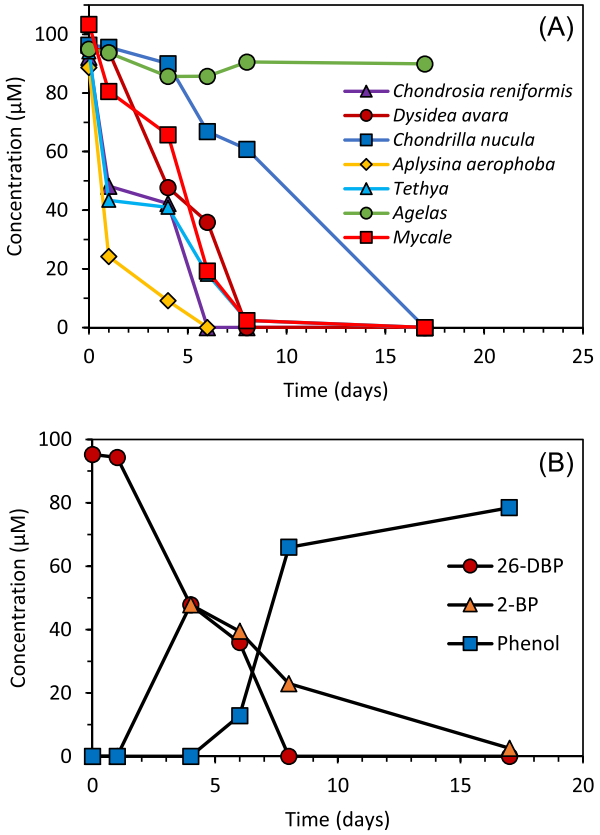

Figure 1. Reductive debromination of 2,6-dibromophenol by sponge-derived enrichment cultures. Loss of 2,6-DBP observed in second generation enrichment cultures of seven different sponge species collected from Croatia **(A)**; Reductive debromination to 2-BP and phenol observed in a culture enriched from a *Dysidea avara* sponge **(B)**.

Results

Widespread distribution of anaerobic debrominating activity in marine sponges

We enriched for bromophenol dehalogenating bacteria from a range of sponge species collected at different sites in the Mediterranean, Atlantic and Pacific, and applied an integrated cultivation and molecular analysis-based approach to analyze the communities. Dehalogenating activity was observed in the majority of anaerobic enrichments from sponge species sampled at all geographical locations tested (Table 1). Debromination of 2,6-DBP to phenol was observed within 2 to 3 weeks in most of the sponge-derived anaerobic cultures, including Mediterranean samples of *Aplysina*, *Axinella*, *Chondrilla*, *Chondrosia*, *Crambe*, *Dysidea*, *Ircinia*, *Mycale* and *Tethya*, and Atlantic samples of *Haliclona*, *Halichondria*, and *Microciona*. An example of debromination activity in sponge enrichment cultures (1/10 transfers of the original sponge cultures) is shown in Fig. 1. No loss of 2,6-DBP or formation of dehalogenated products was observed in sterile controls (data not shown), confirming that dehalogenation was biotic. Stable dehalogenating cultures were maintained by sequential transfers into anaerobic MSM with 2,6-DBP as the electron acceptor and lactate as electron donor and carbon source. Select cultures were also tested with other halogenated phenols, with dehalogenation of 2,4,6-tribromophenol and 2- and 4-iodophenol observed in anaerobic cultures of different sponge species collected from the Mediterranean and Atlantic (Supplementary Table S1).

Desulfoluna spp. detected in dehalogenating cultures

Dehalogenating sponge cultures were screened for the presence or absence of *D. spongiiphila* with two sets of specific primers targeting regions of the 16S and 23S rRNA genes, respectively. PCR analysis detected PCR products of the correct sizes in most of the sponge cultures tested, indicating the presence of bacteria related to *D. spongiiphila* (Table 1). *Desulfoluna* spp. was also detected by PCR in the aquarium grown *Chondrilla nucula* and *Pseudaxinella lunaecharta* sponges (data not shown). Phylogenetic analysis of the PCR amplicons indicated close matches to *D. spongiiphila* and *D. butyraroxidans* (data not shown).

Enrichment of dehalogenating bacteria was observed over successive transfers on bromophenols. TRFLP and DGGE profile analysis provided further evidence for the presence of bacteria highly similar to *D. spongiiphila* in over 20 different sponge species obtained from temperate and tropical oceans (see Supplementary data, Figures S1, S2, S3). Specifically, a TRF (195 bp) or a DGGE band matching *D. spongiiphila* AA1 became progressively more enriched in the debrominating cultures. After successive transfers, a single bacterium became dominant and these cultures were used for more detailed phylogenetic analyses. Other anaerobic bacteria, including sulfidogens such as *Desulfovibrio* spp, were detected by sequence analysis of DGGE bands (data no shown) in some of the cultures, but none could be conclusively linked to dehalogenation activity in the absence of *Desulfoluna* spp. enrichment.

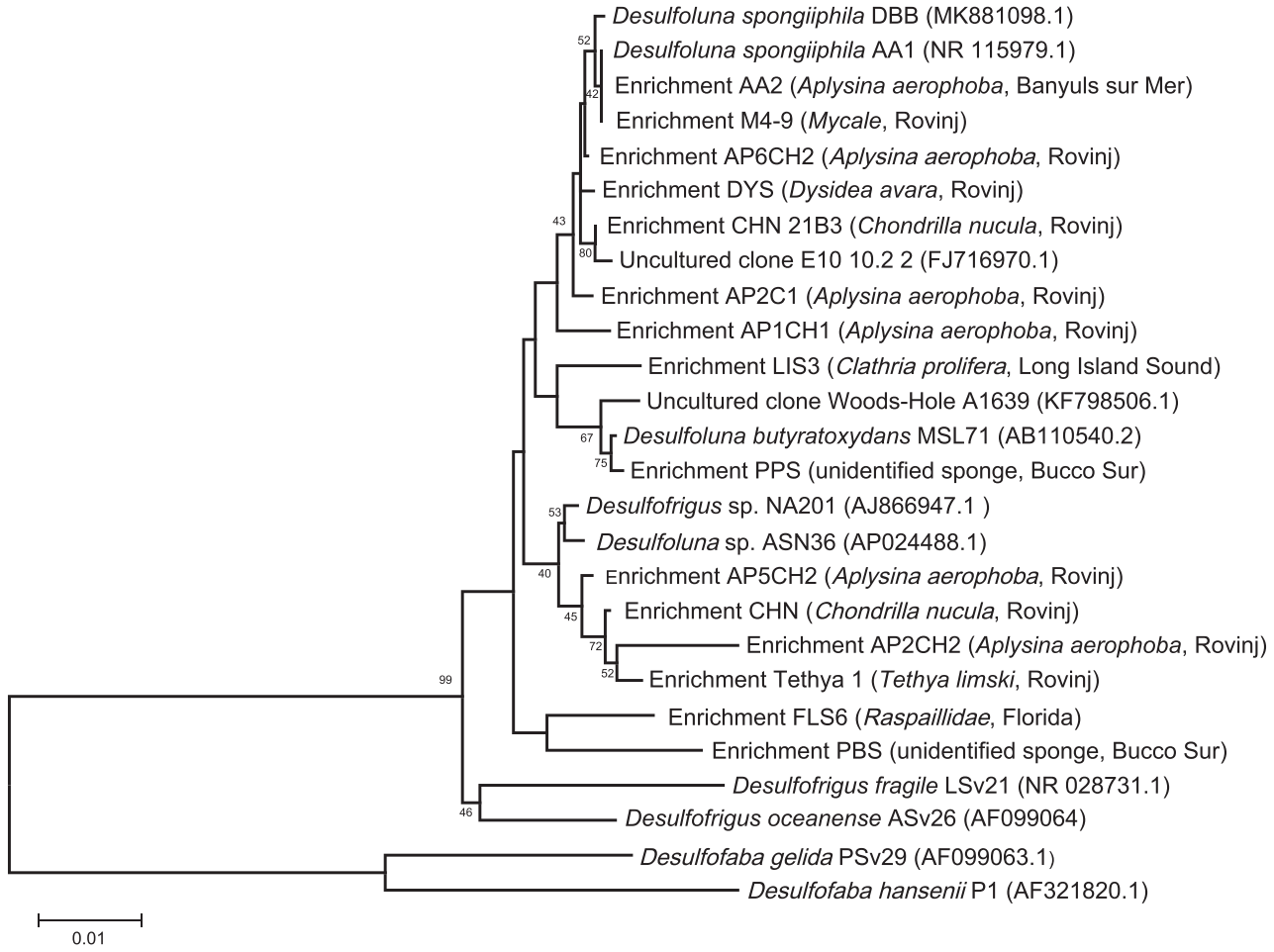

Figure 2. 16S rRNA gene phylogenetic tree comparing dehalogenating isolates, PCR amplicons from sponge enrichment cultures and closely related bacterial species. Sponges were from Banyuls Sur Mer (France), Rovinj (Croatia), Bucco Sur, Tumbes (Peru), Long Island Sound (CT, USA), and deep-water environments of Florida (USA). All positions containing gaps and missing data were eliminated. There were a total of 650 positions in the final dataset. The evolutionary history was inferred using the Minimum Evolution method with 1000 bootstrap replications. Bootstrap values above 40% are shown. Evolutionary analyses were conducted in MEGA11.

Phylogenetic analyses of debrominating Desulfoluna spp.

After initial screening by 16S/23S rRNA gene amplicon analysis (Table 1), a partial 16S rRNA gene amplicon of approximately 800 bp of the various cultures was used to identify the bacteria enriched on 2,6-DBP. Three highly enriched anaerobic debrominating bacterial cultures were obtained via selection from agar shake-tube cultures from enrichments of a *Chondrilla nucula* sponge (CHN) from the Mediterranean, and from two unidentified sponges from the Southeastern Pacific, designated PPS and PBS. Phylogenetic analysis of the 16S rRNA genes (Fig. 2) demonstrated that the debrominating sponge cultures contained bacteria closely related to *Desulfoluna* and which formed a clade separated from the nearest genus *Desulfofrigus* within the *Desulfobacteraceae*. Several of the enrichment cultures from the Mediterranean clustered most closely with *D. spongiiphila* strains AA1 and DBB. Culture PPS from an unidentified sponge off the Peruvian coast was closely related to *D. butyratoxydans*. The two other highly enriched debrominating cultures, CHN and PBS, were more distant to both groups. Overall, the phylogenetic analysis of the sponge enrichments shows diversity within the *Desulfoluna* genus.

In vivo dehalogenation

Dehalogenation of 2,6-DBP into its metabolites (2-BP and phenol) was observed in the tropical sponges *Chondrilla nucula* and *Pseudaxinella lunaecharta* and in three *Clathria prolifera* sponges incubated in seawater (Fig. 3). 2,6-DBP debromination activity by the intact *P. lunaecharta* sponge was observed within the first day of incubation and by day 2 in the *C. nucula* sponge incubations. At day 4, 2,6-DBP was still present in the culture tanks of both sponges. Sampling was stopped when the sponges displayed necrosis at 7 days. All three *C. prolifera* sponges completely debrominated 2,6-DBP to phenol within 2 to 7 days with transient formation of 2-bromophenol and accumulation of phenol, which was eventually depleted. 2-BP was detected between the days 2 and 6 at a concentration of ~50 μM and phenol at a concentration of 300 to 500 μM which then began to decrease. No loss of 2,6-DBP was observed in seawater-only controls indicating the debromination was mediated by the sponge and/or associated microorganisms.

Discussion

This is the first report on the wide global distribution of dehalogenating bacteria in marine sponges and expands on our

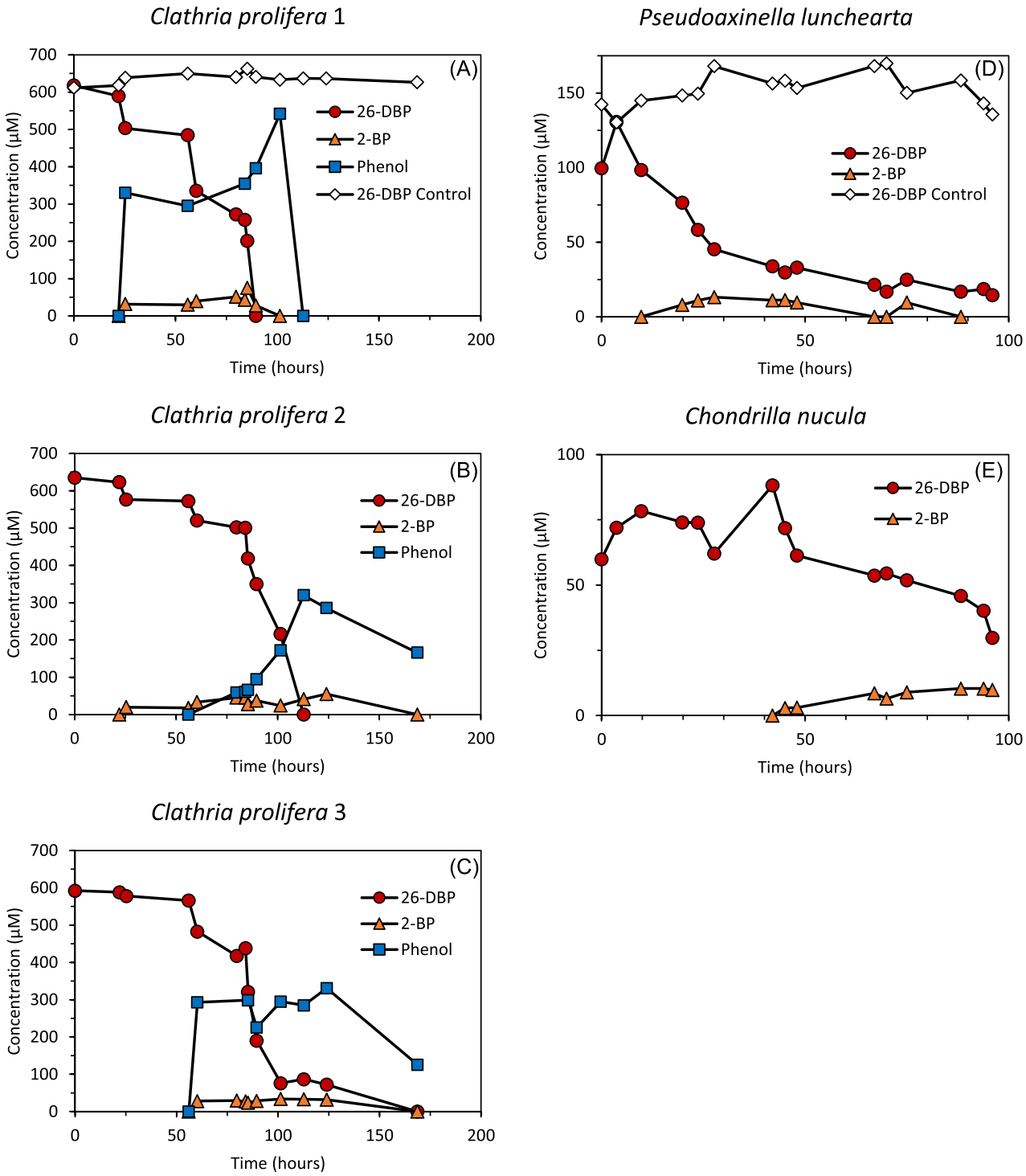

Figure 3. Dehalogenation by three *Clathria prolifera* **(A, B, C)**, a *Pseudaxinella lunaecharta* **(D)**, and a *Chondrilla nucula* **(E)** sponges *in vivo*. Each of the live sponges were incubated in aerated seawater amended with 2,6-dibromophenol.

earlier observation in the Mediterranean sponge *Aplysina aerophoba* (Ahn *et al.* 2003). Dehalogenating bacteria in the sponge animal are thought to be enriched by organohalides such as the brominated tyrosine derivatives, aerophobin-2, aplysinamisin-1, and isofistularin-3, found in *A. aerophoba* (Teeyapant *et al.* 1993, Turon *et al.* 2000). Marine sponges are a generally a source of numerous biogenic organohalides (Gribble 2003), such as indoles (Beukes *et al.* 1998), phenol derivatives (Utkina *et al.* 1998), diphenyl ethers (Bowden *et al.* 2000, Utkina and Denisenko 2006), dioxins (Utkina *et al.* 2001) and polybrominated diphenyl ethers (Carte and Faulkner 1981). This strongly suggests that microbes associated with marine sponges have adapted to organohalides and that members of the sponge microbiota likely can use these compounds to their benefit and/or survival. The sponge-associated microbiota is thus, in part, driven by the organohalogen chemistry of the sponge animal.

Anaerobic enrichment cultures from sponges in temperate and tropical oceans exhibited reductive dehalogenating activity (Fig. 1, Table 1). Enrichment with 2,6-DBP revealed that members of *Desulfoluna* are cosmopolitan debrominating bacteria widely distributed in marine sponges (Fig. 2). Bacteria closely related to *D. spongiiphila* are distributed among species of *Aplysina*, *Agelas*, *Axinella*, *Chondrilla*, *Chondrosia*, *Crambe*, *Dysidea*, *Ircinia*, *Mycale*, and *Tethya* from the Mediterranean, and *Raspaillidae* and *Clathria* in the Atlantic. *D. spongiiphila* strain AA1 was previously isolated from an *Aplysina aerophoba* sponge collected at Banyuls sur Mer (Ahn *et al.* 2009), while *D. spongiiphila* strain DBB was obtained from shore sediment collected in L'Escala, Spain (about 70 km from Banyuls sur Mer) (Peng *et al.* 2020). *D. butyratoxydans* strain MSL71 isolated from estuarine sediment of the coast of Japan (Suzuki *et al.* 2008) is also capable of reductive debromination (Peng *et al.* 2020). While these observations demonstrate a wide distribution of dehalogenating *Desulfoluna* spp., our data does not yet demonstrate whether a particular *Desulfoluna* spp. is specific to a particular sponge species or geographical location. *Desulfoluna* spp. have also been found in other marine sediment samples (Kraft *et al.* 2013) and in a brackish lake (Fig. 1). All sponge enrichments and isolates from a group separate from the next closest genus, *Desulfofrigus*. We were unfortunately unable to maintain the highly enriched cultures CHN, PPS, and PBS for more detailed analysis of their phylogeny and organohalogen metabolism. Based on the partial 16S rRNA gene phylogeny *Desulfoluna* appears to comprise multiple species, however, further work such as whole genome comparison is needed to determine the taxonomy. Interestingly, the capacity for organohalide respiration appears to be widely distributed in other members of marine *Desulfobacterota*, with reductive dehalogenase genes found in approximately 10% of investigated genomes with dehalogenating activity confirmed through functional assays and transcript analysis in select isolates (Liu and Häggblom 2018). Intriguingly, however, there appears to be a sponge-specific selection for *Desulfoluna* spp. from among all the diverse dehalogenating members of the phylum *Desulfobacterota* present in the ocean.

Although sponges process large volumes of oxidized seawater many sponges alternate in redox conditions between periods of high and low water-pumping (Hoffmann *et al.* 2005, Vogel 2008) and can therefore provide a favorable environment for obligate anaerobic bacteria. Indeed, many strictly anaerobic bacteria are routinely found in sponges. For example, in the sponge *Geodia barretti*, portions of the sponge exhibited dramatic decreases in oxygen during low pumping activity (Hoffmann *et al.* 2005) generating an anoxic micro-environment. This depletion of oxygen during low water circulation probably results from the active respiration of a large number of aerobic bacteria present in the mesohyl. We demonstrated *in vivo* debromination by live sponge animals (*Chondrilla nucula*, *Pseudaxinella lunaecharta*, and *Clathria prolifera*) incubated in seawater in this study, suggesting that anaerobic pockets in the animal body provide favorable conditions for the growth and activity of dehalogenating/sulfate-reducing *Desulfoluna*. Organobromine-rich sponges may thus be providing a specialized habitat for organohalide-respiring anaerobic bacteria that mediate a cycling of organohalide compounds within the sponge animal. Reductive debromination of 2,6-DBP by sponge-associated bacteria resulted in measurable carbon isotope fractionation indicating that compound specific isotope analysis might be used to assess reductive debromination and to monitor and estimate *in vivo* dehalogenation in a sponge animal (Horna-Gray *et al.* 2020). 2-Haloacid degrading bacteria have been isolated from the sponge *Hymeniacidon perlevis* (Huang *et al.* 2011) and a reductively dehalogenating bacterium, *Vallitalea guaymasensis* of the order *Clostridiales*, was enriched on 2,4,6-triiodophenol from the marine sponge *Hymeniacidon sinapium* (Kawamura *et al.* 2021). Dehalogenation by these two sponge-associated bacteria was not coupled to respiration. Dehalogenating bacteria have also been found in association with hemichordates that have an incredible capacity to produce organobromine compounds, including bromophenols and bromopyrroles, apparently as defensive agents against predation (King 1988, Boyle *et al.* 1999, Watson *et al.* 2000). This natural and ancient presence of organohalogens in marine environments thus appears to have primed development of various types of microbial dehalogenation (Zanaroli *et al.* 2015, Atashgahi *et al.* 2018).

The halogenated metabolites produced by sponges have likely primed the selection of microbes with the ability to utilize these as carbon sources or electron acceptors for growth. While this association with dehalogenating bacteria may be ancient, it is not clear whether dehalogenating bacteria provide any benefit to the sponge host. To date, several types of microbe-sponge associations, from opportunistic to strict vertical symbioses, have been identified (Taylor *et al.* 2007). In certain sponges, vertical transmission of microorganisms from the sponge to its larvae has been demonstrated (e.g. Enticknapp *et al.* 2006, Sharp *et al.* 2007). The detection of these bacteria at early stages in the life of a sponge suggests that these symbionts are needed for their survival. If *Desulfoluna* spp. are opportunistic microorganisms that find a suitable environment in sponges, then we should be able to isolate these species also from seawater and sediments. Indeed, *D. spongiiphila* strain DBB and *D. butyratoxydans* strain MSL7 were isolated from sediments. Future work will examine whether anaerobic debrominating bacteria form stable or dynamic populations within the sponge animal, how the dehalogenating bacteria are acquired, and whether the dehalogenating strains/species are specific to a particular sponge host. Additionally, the diversity of RDases found in sponge-associated *Desulfoluna* spp. and their ability to dehalogenate different natural and anthropogenic organohalides need to be examined. This will expand our knowledge of reductive dehalogenation in marine environments and its possible ancient involvement with marine sponges.

In conclusion, we show that *Desulfoluna* spp. form a cosmopolitan group of dehalogenating bacteria distributed in organohalide-containing sponges across different geographic locations. These sponge-associated dehalogenating bacteria are active *in vivo* and are expected to impact the fate of both natural and man-made brominated organics in the ocean. Sponges can be regarded as natural microbial bioreactors and are thus an excellent model system to study an organohalogen cycle within a confined and stable environment.

Supplementary Data

Supplementary data are available at FEMSEC online.

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