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Evidence for free-living *Bacteroides* in *Cladophora* along the shores of the Great Lakes

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ABSTRACT: *Bacteroides* is assumed to be restricted to the alimentary canal of animals and humans and is considered to be non-viable in ambient environments. We hypothesized that *Bacteroides* could persist and replicate within beach-stranded *Cladophora glomerata* mats in southern Lake Michigan, USA. Mean *Bacteroides* concentration (per GenBac3 Taqman quantitative PCR assay) during summer 2012 at Jeorse Park Beach was 5.2 log calibrator cell equivalents (CCE) g^{-1} dry weight (dw), ranging from 3.7 to 6.7. We monitored a single beach-stranded mat for 3 wk; bacterial concentrations increased by 1.6 log CCE g^{-1} dw and correlated significantly with ambient temperature ($p = 0.003$). Clonal growth was evident, as observed by >99% nucleotide sequence similarity among clones. In *in vitro* studies, *Bacteroides* concentrations increased by 5.5 log CCE g^{-1} after 7 d (27°C) in fresh *Cladophora* collected from rocks. Partial sequencing of the 16S rRNA gene of 36 clones from the incubation experiment showed highly similar genotypes (≥97% sequence overlap). The closest enteric *Bacteroides* spp. from the National Center for Biotechnology Information database were only 87 to 91% similar. Genomic similarity, clonality, growth, and persistence collectively suggest that putative, free-living *Bacteroides* inhabit *Cladophora* mats of southern Lake Michigan. These findings may have important biological, medical, regulatory, microbial source tracking, and public health implications.

KEY WORDS: Anaerobic environments · *Bacteroides* · *Cladophora* · Sequencing · Uncultured bacteria

INTRODUCTION

Bacteroides are mutualistic, gram negative, non-spore-forming bacteria that are well represented in the intestinal microflora of mammals, with cell densities as high as 10^{10} to 10^{11} g^{-1} feces in humans (Madigan et al. 2003). While mutualistic, *Bacteroides* may become opportunistic pathogens; several species within the *Bacteroides fragilis* group have been linked to various diseases and clinical manifestations, such as bacteremia and colonic, skin and soft-tissue infections (Wexler 2007). Although there are about 20 described species of *Bacteroides* (Shah & Collins 1989, Wexler 2007), it is generally believed that many more species remain undescribed due to culturing difficulties. Species of unknown viability and origination are listed in the National Center for Biotechnology Information (NCBI) database from various environments, such as sludge, rivers, sediments, and biofilms; we know of no species found that are unique to any environmental matrix. *Bacteroides* are strict anaerobes, but some tolerance to low oxygen concentrations has been noted for select species within the *B. fragilis* group (Baughn & Malamy 2004).

There are many environmental habitats that can be characterized as anaerobic, aphotic, and warm, with adequate nutrients (glycans) for *Bacteroides* suste-

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nance. *Bacteroides* are efficient users of polysaccharides of complex carbohydrates (see review by Hooper et al. (2002)), and previous laboratory studies have shown that they can utilize polysaccharides associated with algae (Michel & Macfarlane 1996, Bakunina et al. 2012, Hehemann et al. 2012). High levels of other fecal indicators and some pathogens, such as *Clostridium perfringens*, *C. botulinum*, *Escherichia coli,* enterococci, *Campylobacter*, and *Salmonella*, are common in *Cladophora* mats in the Great Lakes (Byappanahalli et al. 2003, Whitman et al. 2003, Byappanahalli & Whitman 2009, Verhougstraete et al. 2010).

The objectives of this study were to determine whether conditions in *Cladophora* mats promoted *in situ* and *in vitro* replication of *Bacteroides* and to identify strain(s) most likely to thrive in these algal mats. We used *Cladophora* as a model to test whether *Bacteroides* can develop viable populations in nutrient-rich, anaerobic algal matter, and we discuss the biological, public health, and environmental implications of our findings.

MATERIALS AND METHODS

Study area

Jeorse Park (JP) Beach is located on the southwest shore of Lake Michigan in East Chicago, Indiana, USA (41.651110° N, 87.433422° W), in a highly industrialized area. The beach is bounded by 2 breakwalls, which act as substrates for *Cladophora* growth and also help to direct algae along the shoreline where they accumulate in shallow water or become stranded on the beach.

For one *in vitro* growth experiment, we collected attached algae from a breakwall at Portage Lakefront and Riverwalk (PL), an Indiana Dunes National Lakeshore beach, located in Portage, Indiana, USA (41.631170° N, 87.179369° W). All other sampling and experiments occurred at JP.

Sample collection

Between 27 June and 25 September 2012, samples from stranded algal mats were collected at varying frequencies, from 1 to 3 times a week for a total of 15 sampling occasions. Due to the transient nature of *Cladophora* mats along the shore, the sampling location varied somewhat (<150 m). Photos of the algal mats were taken frequently to document their temporal duration; it was established that between 21 August and 11 September the same mat was present on the Jeorse Park shore. *Cladophora glomerata* (L.) Kutz dominated the mats (95%), but incidental *Chara*, *Spirogyra*, and *Mougeotia* occurrence was noted. Sampling occurred before 11:00 h CDT to reduce solar insolation effects and interference from beach visitors. Triplicate samples (200 to 400 g wet weight) were collected with nitrile-gloved hands and placed into sterile polyethylene bags. Samples were transported to the laboratory in a cooler on ice, stored at 4°C, and processed within 24 h. Continuous measurements of air temperature were collected from an on-site weather station located at 63rd Street Beach, Chicago, Illinois, ~20 km NNW of the study beach. Means of the highest and lowest temperature readings within the 24 h period were calculated.

In vitro incubation studies

For an *in vitro* growth study (incubation study I), *Cladophora* was collected in September 2012 at PL. Approximately 2 kg (wet weight) of algae attached to rocks was collected. The *Cladophora* was hand-mixed in a sterile container, and five 1 l sterile bottles were each filled with 400 g. With the exception of time 0 (D0), bottles were incubated in the dark at 27°C; caps were loose-fitting to allow gas exchange. Samples were analyzed at D0 and after 7, 14, 21, and 28 d of incubation (D7, D14, D21, and D28). Two small containers filled with water were kept in the incubator to minimize moisture loss from the samples.

To demonstrate reproducibility, a second *in vitro* experiment (incubation study II) was performed, in which *Cladophora* samples from the seasonal JP collection, which had been stored at –20°C, were thawed and used. Samples from 3 distinct time periods were chosen: 27 June, the first decaying mat of the season; 21 August, the first day of a long-term mat; and 30 August, the peak concentration of *Bacteroides* quantitative PCR (qPCR) calibrator cell equivalents (CCE) during the season. Three replicates representing each date were individually homogenized with a sterile spatula for 1 min, and subsamples (10 g) were transferred into 15 ml tubes (3 tubes per replicate, total of 27 tubes). Tubes were covered with parafilm tape, and a pin-pricked hole allowed gas exchange; all tubes, with the exception of D0, were incubated at 27°C in the dark. Samples were analyzed at D0 and after 3 and 9 d of incubation

(D3 and D9). A beaker filled with water was placed in the incubator to minimize sample moisture loss during incubation.

Sample processing

Seasonal

Sample replicates were homogenized with sterile spatulas, and subsamples (25 g) were placed into dilution bottles and combined with 100 ml phosphate-buffered water (PBW; pH 7.0 ± 0.2) containing 0.01% hydrolyzed gelatin (Ishii et al. 2006). Samples were shaken twice for 2 min 30 s, with a 1 min resting period in between; the mixtures were then transferred to sterile 50 ml centrifuge tubes and centrifuged (622 × *g*) for 1 min. The resulting supernatants were decanted into sterile dilution bottles, aliquots (40 ml) from the 3 replicates were combined, and 15 ml of the composite was filtered through 0.4 µm polycarbonate membranes for qPCR analyses (see 'qPCR assay' below). Sample wet-to-dry-weight ratios were determined individually after drying algal subsamples (5 g) at 105°C for 24 h, and the mean ratio was used for calculating cell densities g^{-1} dry weight (dw).

In vitro incubation studies

For incubation study I, 300 ml of PBW containing 0.01% hydrolyzed gelatin was added to each of the 5 bottles containing 400 g algae and placed on an orbital shaker for 30 min at 200 rpm. The liquid extracts elutriated from algae were transferred to 50 ml centrifuge tubes and centrifuged at 622 × *g* for 1 min; the supernatants were then decanted into new tubes to be used for further processing. For incubation study II, the sample tubes were thawed and the contents emptied into sterile dilution bottles; 50 ml of PBW containing 0.01% hydrolyzed gelatin was added to each bottle. Samples were then shaken and centrifuged using the same procedure described previously under the seasonal samples.

Molecular analyses

For molecular analysis, aliquots of extracts (5 to 15 ml) elutriated from algae were filtered through 0.4 µm polycarbonate filters (Millipore). Filters were immediately stored in 2 ml semi-conical, screw cap centrifuge tubes and stored at –80°C until processing.

DNA extraction

Filters were thawed and transferred to PowerBead tubes, and DNA was extracted using the PowerSoil® DNA extraction kit (MO BIO Laboratories) as per the manufacturer's instructions, with the following modifications: to each tube, 11.8 µl of salmon testes DNA (Sigma-Aldrich), 0.2 µg ml^{-1} final concentration, was added to the supernatant following the bead-beating step as an internal control and reference for qPCR amplification. Testing has shown (Powersoil® only) that adding the internal control pre- and post-bead-beating step showed no significant difference ($p = 0.215$) between the threshold cycle number (C_t) reference values (data not shown). Additionally, the final DNA elution step was repeated 3 times with 3 aliquots of elution buffer (30, 30, and 40 µl).

The primers for PCR and qPCR assays in this study have been used previously to identify and characterize members within the order *Bacteroidales* (Bernhard & Field 2000, US Environmental Protection Agency 2010). However, in the present study, we refer to our target bacteria as *Bacteroides*, since the BLAST analyses repeatedly confirmed that our sequences had similarities (in excess of 96%) with those listed in the NCBI database originating from clinical or environmental samples, with the majority of them designated as genus *Bacteroides* from uncultured bacteria.

qPCR assay

Bacteroides densities in samples were determined using qPCR, as described elsewhere (US Environmental Protection Agency 2010). Amplification reactions were performed using a Bio-Rad CFX Connect instrument (Bio-Rad) in 96-well PCR plates, with a reaction volume of 25 µl containing 5 µl of 5× or 25× diluted DNA template. *B. thetaiotaomicron* (ATCC 29741) was used for developing a standard curve and calibrators. *Bacteroides* in test samples were quantified as CCE. Appropriate positive and negative controls, *B. thetaiotaomicron*, no templates (PCR water), and extraction blanks were included in all assays. Because of the complex nature of the samples tested in this study, we were not able to sufficiently decrease PCR inhibitors on certain samples from incubation studies (27% of the total number of samples analyzed), as indicated by salmon reference values. Nonetheless, we are confident that the detected trends in CCE values are reliable, and the results were reproducible across experiments conducted in this study.

PCR assay and cloning

PCR primers Bac32F and 708R were used to target an ~700 bp region of the 16S rRNA gene as previously described (Bernhard & Field 2000). Briefly, reactions were carried out in a 25 µl reaction mixture containing the following components: 1 × PCR buffer, 1.5 mM $MgCl_2$, 16 µg bovine serum albumin, 200 µM deoxynucleoside triphosphates, 0.25 µl *Taq* Polymerase (i.e. 5.0 U μl^{-1}), 0.35 µM each primer (final concentration), and 2 µl DNA template. The assay thermal cycling conditions were as follows: 3 min at 94°C, followed by 35 cycles of 60 s at 94°C, 45 s at 53°C, 45 s at 72°C, and a 7 min final extension at 72°C. PCR amplifications were carried out on a Bio-Rad T100 Thermal Cycler (Bio-Rad), and PCR products were separated on a Lonza FlashGel® System (agarose gel, 2.2% w/v; Lonza Rockland). Following gel electrophoresis, PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). PCR products from 10 algal samples were chosen for sequencing: 6 from seasonal algal samples and 4 from incubation study I (D0, D7, D14, and D28).

Purified PCR products of D0 and D7 algal samples were cloned into the pGeM®-T Easy vector (Promega) according to the manufacturer's protocol. The vectors were then transformed into *Escherichia coli* cells (JM109 high efficiency). From each D0 and D7, 24 transformants were randomly selected and inoculated into 1 ml Luria-Bertani freezing medium. The tubes were then incubated overnight on an orbital shaker (100 rpm) at 37°C. 100 µl aliquots were sent to the Genomics Core at Michigan State University for further processing and sequencing. *Bacteroides thetaiotaomicron* (ATCC 29741) served as our reference and control for all analyses.

Phylogenetic analysis

Sequencing was carried out using ABI BigDye Terminator v3.1 chemistry and POP-7 polymer, and sequencing reactions were carried out in both directions and run on an ABI Prism 3730xl DNA analyzer (Applied Biosystems). Sequence identity was verified using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/). Three sequences had no significant similarity matches within the GenBank database, and thus, they were removed from the analysis. Trimmed sequences were aligned in MEGA5 using ClustalW. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model (Kimura 1980, Tamura et al. 2011). All positions with <95% site coverage were eliminated. Bootstrap values were obtained from a consensus of 1000 resamplings; bootstrap values <70% were removed.

Statistical analyses

Statistical analyses were performed using SPSS version 12.0 (SPSS 2003). Statistical procedures were performed on log_{10}-transformed CCE data. The Kolmogorov-Smirnov test, a non-parametric test, was used to test parametric assumptions of equality of variance and normal distribution. Pearson correlation (Pearson's r) was reported for *Bacteroides* CCE and air temperature. Analysis of variance with a Tukey post-hoc test was used to examine the significance in *Bacteroides* CCE over time during *in vitro* incubation study II.

RESULTS

Seasonal field sampling

Mean concentrations of *Bacteroides* (log CCE g^{-1} dw ± SE) in algal samples collected throughout the sampling season were 5.21 ± 0.23 (n = 15). The lowest and the highest concentrations, 3.72 and 6.73, were observed on 27 June and 30 August (Fig. 1). The wet-

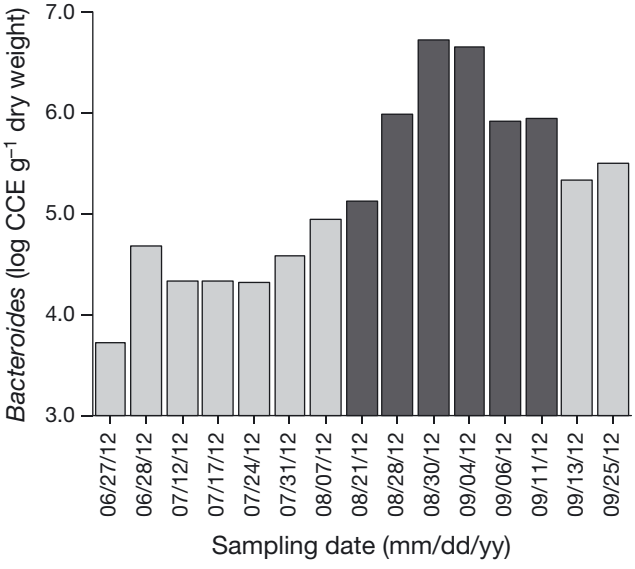

Fig. 1. *Bacteroides* concentrations (log calibrator cell equivalents [CCE] g^{-1} dry weight) eluted from algae collected from Jeorse Park Beach IN, USA (from 27 June to 25 September 2012). Dark gray bars represent samples collected from a stranded mat that persisted over 3 wk

to-dry ratio of the algal mat samples ranged from 2.06 to 11.28. Notably higher concentrations were detected in samples from the same consistent algal mat stranded along the shoreline between 21 August and 11 September (Fig. 1, black bars). Bacterial concentrations were significantly correlated with mean daily air temperature during this time (Pearson r = 0.955, p = 0.003, n = 6).

In vitro incubation studies

The concentration of *Bacteroides* (log CCE g^{-1} wet weight) at D0 of incubation study I was 2.48 and increased to 8.01 at D7 of incubation (Fig. 2a). Concentrations remained at this level for the remaining sampling days (D14 and D28). For incubation study II, concentrations of *Bacteroides* (log CCE g^{-1} dw ± SE) at D0 ranged from 4.25 ± 0.36 (late June samples) to 5.67 ± 0.13 (late August samples) (Fig. 2b–d). In both August samples (21 and 30 August), concentrations increased significantly at D3 of incubation ($p < 0.001$), but concentrations decreased in June samples. At D9, concentrations of *Bacteroides* were significantly higher in all samples relative to D0 measurements ($p < 0.001$), with the highest concentration observed in the 30 August sample: 8.79 ± 0.04. In both incubation studies, there was evidence of inhibition as noted by salmon C_t values (i.e. D7, D14, and D28 from incubation study I; all 3 replicates from D3 and D9 from 27 June sample in incubation study II; 1 replicate each from 21 and 30 August samples). Although the final CCE in these samples may have been affected by the presence of inhibitors, the increasing trend detected over time was conserved.

Sequencing

Bacterial sequences of the samples from our seasonal field sampling were 99% identical. Otherwise, partial sequences of the 16S rRNA gene from seasonal field sampling at JP were related to *Bacteroides* from 2 human and 8 non-human sources. Top matches from the GenBank database included: *Bacteroides* sp. (98 to 99% identity) isolated from a granulated sewage sludge in Japan (GenBank AB787271); uncultured *Bacteroidales* strains (98 to 99% identity) from a wastewater treatment plant in Ireland (EU573848); 6 uncultured *Bacteroidales* strains (97 to 98% identity) from river water impacted by migratory sandhill cranes in Nebraska (KC149761; KC149831; KC149832; KC149834; KC149844; KC149845); uncultured *Bac-*

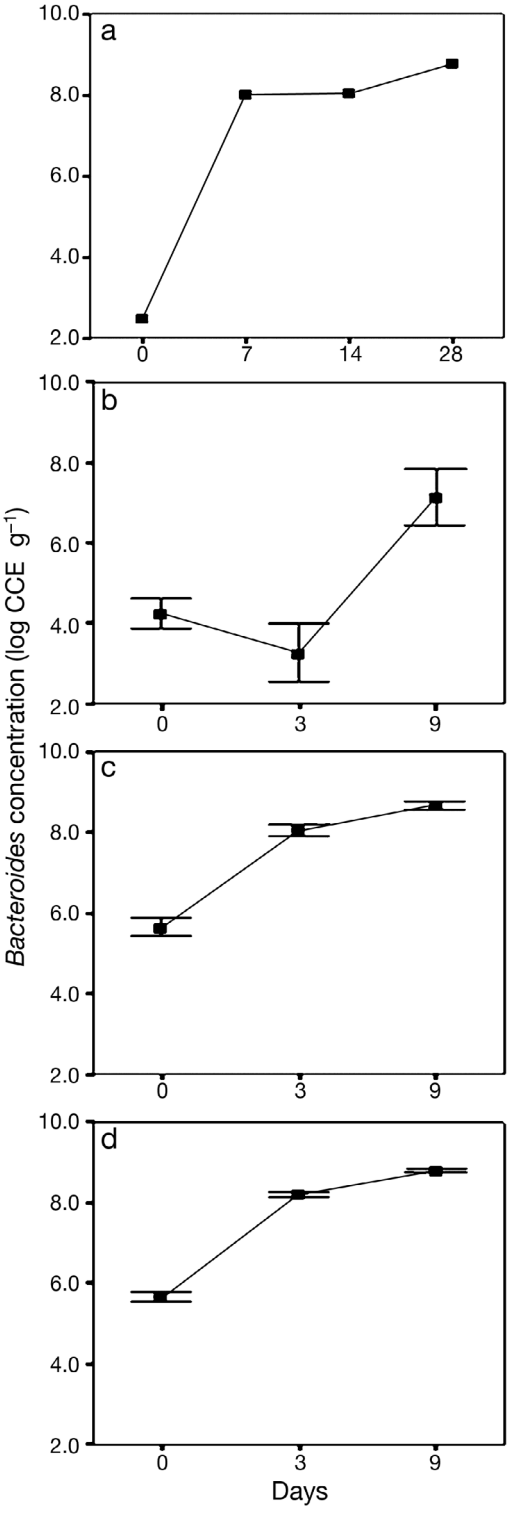

Fig. 2. (a) *Bacteroides* concentrations (log calibrator cell equivalents [CCE] g^{-1} wet weight) within *Cladophora* from Portage Lakefront Beach, IN, USA, on 17 September 2012 (incubation study I). (b–d) Log mean ± 1 SE *Bacteroides* concentrations (log CCE g^{-1} dry weight) within *Cladophora* for samples taken on 27 June, 21 August, and 30 August 2012, respectively, at Jeorse Park Beach, IN, USA, (incubation study II)

teroides sp. (98% identity) from biofilm samples from a cave in Tennessee (JN820138); and uncultured bacteria belonging to *Bacteroidales* (98% identity) from river water in Korea (GQ921905).

The 36 clones included in sequencing analyses from *in vitro* incubation study I were 98 to 100% identical to one another; however, 3 clusters were still observed: CLADOPHORA A (20 clones), CLADOPHORA B (5 clones), and CLADOPHORA C (11 clones) (Fig. 3). Top matches from the GenBank database for bacterial sequences of D0 and D7 clones from *in vitro* incubation study I were nearly identical (97 to 99% similarity) to sequences from seasonal field sampling at JP. Bacterial sequences from D7, D14, and D28 of *in vitro* incubation study I were 99% identical among one another. Of the 21 *Bacteroidales* sequenced in the initial population, 24% matched *Prevotella*, but none were detected thereafter.

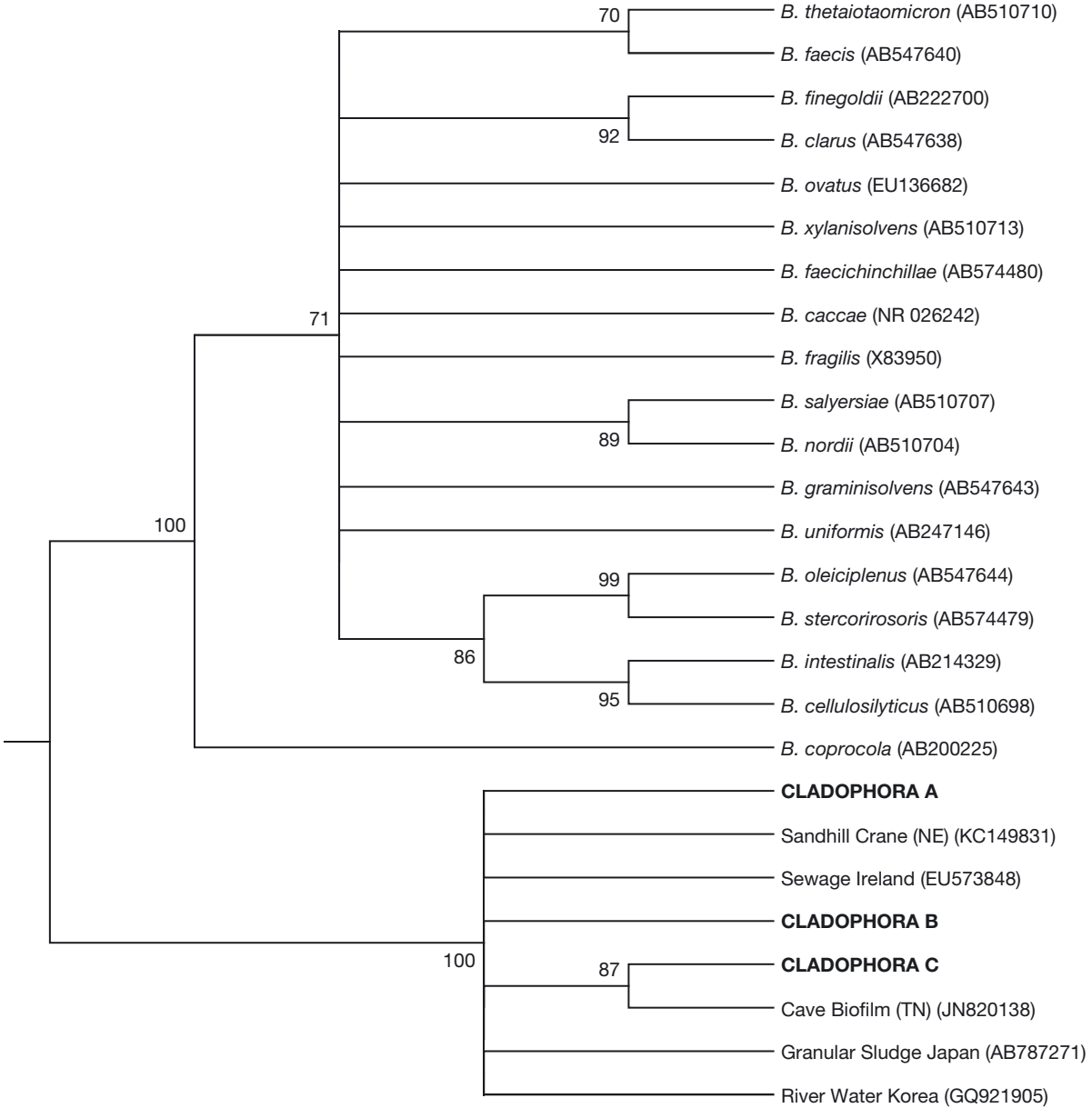

Fig. 3. An unrooted phylogenetic tree derived from partial 16S rRNA gene sequences (540 positions) obtained from *Bacteroides* spp. amplified from *Cladophora* samples. *Cladophora* A, B, and C represent a total of 36 clonal sequences. Sequences obtained from the GenBank database are designated by the species name and/or accession number. Bootstrap values are shown as percentages of 1000 trees; values <70% are omitted

DISCUSSION

Bacteroides is most commonly recognized as a commensal organism living in the mammalian enteron, and it is not generally considered free-living or capable of surviving more than a few hours in the ambient environment (Kreader 1998, Falkow et al. 2006, Layton et al. 2006, Ballesté & Blanch 2010). We found no previous studies demonstrating that the bacteria were free-living and only a few studies suggesting that *Bacteroides* can replicate in non-enteric habitats. Walters & Field (2006) detected actively growing *Bacteroidales* in laboratory mesocosms, and van der Wielen & Medema (2010) acquired circumstantial evidence that gene copies of *Bacteroides* increase during pre-treatment of ground water. In this study, however, we followed viable populations both in natural and laboratory conditions over an extended period of time; these results may be the first evidence of sustained ambient multiplication of an environmentally occurring *Bacteroides*.

Growth

We observed a steady increase of 1.6 log CCE g^{-1} dw of clonal *Bacteroides* over 8 d in a single, persistent mat in August 2012, suggesting *in situ* growth rather than merely DNA accumulation from wildlife or sewage. BLAST analysis of our sequences showed a 99% similarity in genomic identity among these populations with the homogeneity maintained at this level, suggesting clonality of these *Cladophora*-associated *Bacteroides*. Environmentally, we found no physical conditions that would limit the growth of these bacteria: the ambient high temperature was 26°C with a low of 19°C; the surface temperature of the algal mat reached as high as 34°C (Fig. 4); *Cladophora* moisture content was 76% w/w, with the stranded algae likely turning anaerobic within 1 h *in vitro* (J. Peller et al. unpubl.); 28.5% of the algae is made up of microfibrils of glucose, galactose, arabinose, and xylose (Wyatt et al. 2014). Seaman (2009) reports 359 mg l^{-1} NH_4-N, 3.8 mg l^{-1} NO_3-N, 5.5 mg l^{-1} available P, and 366 mg l^{-1} available K in compost *Cladophora*. In our pilot experiments, concentrations of seeded *B. thetaiotaomicron* in decayed algae increased after 4 to 9 d of incubation in 26 to 35°C (data not shown), suggesting that *Cladophora* may be a suitable growth medium for this species as well.

Our observations at JP beach were followed by 2 laboratory validations of *Bacteroides* growth. A 5.5 log CCE g^{-1} wet weight increase occurred at 27°C at D7 (incubation study I) and a 3 log CCE g^{-1} dw increase at D3 (incubation study II), with concentrations reaching 8 to 9 log CCE g^{-1} in both experiments, along with very low genomic diversity (sequence similarity >97%). Despite the very high concentrations detected by qPCR, we were unsuccessful at isolating and culturing this presumably unknown *Bacteroides* using formulated media.

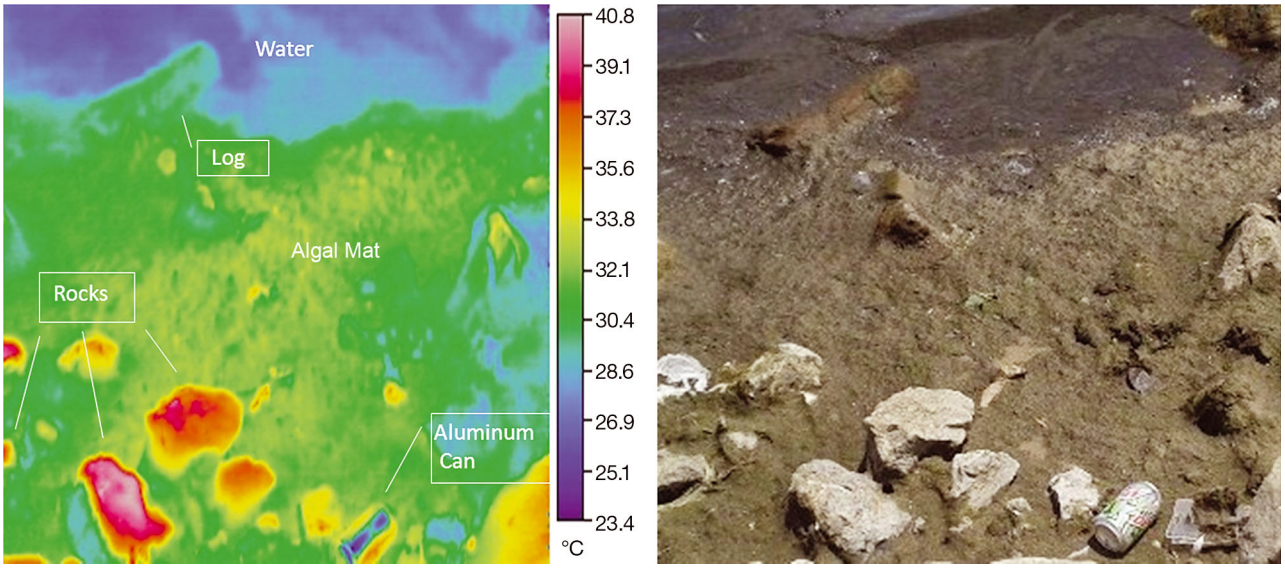

Fig. 4. Thermal (left) and digital (right) images of a portion of large mat of stranded *Cladophora* extending several hundred meters along the shore at Jeorse Park Beach IN, USA, on 27 June 2012. Note that the algae are detached and in advanced stages of decomposition

Persistence

Short- to longer-term persistence of viable *Bacteroides* have been noted, principally in sewage and animal feces (e.g. Okabe & Shimazu 2007, Bell et al. 2009, Walters & Field 2009, Ballesté & Blanch 2010, Liang et al. 2012). Most studies on *Bacteroidales* have been done in surface and groundwater environments. Some research, however, has been done in specialized microhabitats, such as Alpine soils (Vierheilig et al. 2012), shellfish samples from France (Mauffret et al. 2013), thick biofilm inside influent pipes (Savichtcheva et al. 2007), groundwater samples from the Netherlands (van der Wielen & Medema 2010), sand (Yamahara et al. 2007, Yamahara et al. 2012, Eichmiller et al. 2013), and sediments (Vogel et al. 2007, LaPara et al. 2011, Eichmiller et al. 2013). In a detailed study by Olapade et al. (2006) of microbial communities associated with *Cladophora* mats in Lake Michigan, it was found that as much as 40% of clonal sequences belonged to the *Cytophaga-Flavobacterium-Bacteroides* group, and the authors concluded that *Bacteroides* may comprise a large portion of bacteria in *Cladophora*. In the present study, *Bacteroides* in stranded algae persisted under ambient conditions for 3 mo under varying hydrometeorological conditions typical of a temperate summer. During the season, *Cladophora* mats remained stranded, often returned to the lake, re-stranded, were occasionally covered in sand, and uncovered by the swash. While we follow *Bacteroides* on these ephemeral mats, we focused on 21 August to 11 September when the mat remained relatively stable and isolated from the lake. The seasonal presence of *Bacteroides* and evident clonality strengthens the belief that these bacteria are capable of maintaining their population through clonal growth; long-term persistence is also evident.

We suggest that *Bacteroides* populations recovered during our seasonal sampling at JP may be of non-enteric origin, since they were only 87 to 91% similar to fecal species of *Bacteroides* (Fig. 3) and the *B. fragilis* group, which is characteristic of human gut flora (Liu et al. 2003, Wexler 2007). Our comparisons with NCBI reference sequences showed 97 to 99% similarity to uncultured *Bacteroides* sp. This finding is supported by Bower et al. (2005) who reported unknown species of *Bacteroides* present in water samples in areas of unknown human waste input. Similarly, *Bacteroides* were detected in deep groundwater and tap water in the Netherlands with no history or evidence for fecal contamination (van der Wielen & Medema 2010). Vierheilig et al. (2012) suggest persistence of some autochthonous, non-intestinal populations detected by AllBac and BacUni primers in Alpine soils, with no indication of human and animal fecal contamination; numbers approached fecal concentrations of 5 to 8 log CCE g^{-1}. Finally, it has been concluded that *Cladophora* mats along beaches of Lake Michigan are comprised mostly of a phenotypically undescribed bacteria community, as 16S rRNA clones were unclassified and uncultured (Olapade et al. 2006).

The genetic similarity of our *Bacteroides* to those found in Korea, Japan, Ireland, and the USA may suggest that this presumed unknown species is cosmopolitan. These findings and the high confidence of clonal growth in the *in vitro* studies (98 to 99% sequence similarity), along with field observations over the summer of 2012, support the hypothesis that at least some *Bacteroides* are capable of growing and persisting in nature outside of an animal host in the presence of *Cladophora*. The wider prevalence and persistence of *Bacteroides* of environmental origin or in such environmental reservoirs is currently not well understood.

Implications

Our proposition that *Bacteroides* can survive and multiply in the environment has important biological, ecological, and public health implications, which raise new questions. Despite the potential for fecal origin of these *Bacteroides*, the phylogenetic relationship with enteric *Bacteroides* is remote, and we do not know whether the strains we observed are *Cladophora*-adapted, ubiquitous, or opportunistic. Other studies have shown that *Bacteroides* persist longer in marine habitats (Green et al. 2011); perhaps the environmental species are even more common in saltwater habitats. We believe that as further investigations are conducted, more examples of free-living *Bacteroides* will be found.

Fecal *Bacteroides* has been suggested as an indicator of wastewater pollution, with particular advantages over current indicators — enterococci and *Escherichia coli* — because (1) enteric *Bacteroides* are abundant in the human gut, and (2) it is generally believed that *Bacteroides* does not survive for long nor multiply in nature. Finding *Cladophora*-associated *Bacteroides* in this study suggests that bacterial pathogens may similarly persist and grow in *Cladophora* mats, potentially exposing swimmers during recreational activities. Thus, health risk studies are needed to better understand this problem since swimmers often encounter the decaying alga on beaches.

This study demonstrates the long-term persistence and growth of *Bacteroides* in decaying *Cladophora* mats of southern Lake Michigan. It suggests that this is a general phenomenon in similar fresh- and marine-water environments with abundant filamentous algae or other nutrient sources. The observation of *Bacteroides* growing both *in situ* and *in vitro* and our genomic analyses supports this conclusion. If true, much work remains in our understanding of the common occurrence of viable environmental *Bacteroides.*

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