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Rapid Enrichment of *Dehalococcoides*-Like Bacteria by Partial Hydrophobic Separation

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ABSTRACT Organohalide-respiring bacteria can be difficult to enrich and isolate, which can limit research on these important organisms. The goal of this research was to develop a method to rapidly (minutes to days) enrich these organisms from a mixed community. The method presented is based on the hypothesis that organohalide-respiring bacteria would be more hydrophobic than other bacteria as they dehalogenate hydrophobic compounds. The method developed tests this hypothesis by separating a portion of putative organohalide-respiring bacteria, those phylogenetically related to *Dehalococcoides mccartyi*, at the interface between a hydrophobic organic solvent and an aqueous medium. This novel partial separation technique was tested with a polychlorinated biphenyl-enriched sediment-free culture, a tetrachloroethene-enriched digester sludge culture, and uncontaminated lake sediment. Significantly higher fractions, up to 20.4 times higher, of putative organohalide-respiring bacteria were enriched at the interface between the medium and either hexadecane or trichloroethene. The selective partial separation of these putative organohalide-respiring bacteria occurred after 20 min, strongly suggesting that the separation was a result of physical-chemical interactions between the cell surface and hydrophobic solvent. Dechlorination activity postseparation was verified by the production of *cis*-dichloroethene when amended with tetrachloroethene. A longer incubation time of 6 days prior to separation with trichloroethene increased the total number of putative organohalide-respiring bacteria. This method provides a way to quickly separate some of the putative organohalide-respiring bacteria from other bacteria, thereby improving our ability to study multiple and different bacteria of potential interest and improving knowledge of these bacteria.

IMPORTANCE Organohalide-respiring bacteria, bacteria capable of respiring chlorinated contaminants, can be difficult to enrich, which can limit their predictable use for the bioremediation of contaminated sites. This paper describes a method to quickly separate *Dehalococcoides*-like bacteria, a group of organisms containing organohalide-respiring bacteria, from other bacteria in a mixed community. From this work, *Dehalococcoides*-like bacteria appear to have a hydrophobic cell surface, facilitating a rapid (20 min) partial separation from a mixed culture at the surface of a hydrophobic liquid. This method was verified in a polychlorinated biphenyl-enriched sediment-free culture, an anaerobic digester sludge, and uncontaminated sediment. The method described can drastically reduce the amount of time required to partially separate *Dehalococcoides*-like bacteria from a complex mixed culture, improving researchers' ability to study these important bacteria.

KEYWORDS dechlorination, *Dehalococcoides*, enrichment, organohalide-respiring bacteria, separation

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Incredible advances in culture-independent methods have provided a glimpse of the microbial diversity and capabilities that exist in the environment (1–3); nevertheless, microbial enrichments and isolates are still critical for advancing knowledge. Enrichments or isolates facilitate better assessment of the physiology, structure, and function of populations within a complex microbial community. In fact, the study of enrichments and isolates has resulted in discoveries as diverse as determining the enzymes responsible for degradation of the pollutant vinyl chloride (4), isolating possible anticancer agents (5), and understanding the cell structures of bacteria within candidate phyla with no cultured representatives (6). Unfortunately, while culturing techniques have improved, they still rely heavily on knowledge of an organism's ideal growth conditions and tend to enrich the fastest growing organisms in a sample (7). Better enrichment methods, which may combine physical and metabolic enrichment techniques, are therefore needed to facilitate the progress of biotechnological applications and enhance basic knowledge.

Differences in cell surface properties may be one way to distinguish among and therefore separate bacteria with specific capabilities from a community. Indeed, bacteria have evolved various cell surface characteristics to allow them to better exploit a particular niche. For example, in the medical field, bacteria that colonized implants were found to have positively charged cell surfaces to promote adhesion to negatively charged implant materials (8). Bacteria capable of degrading hydrophobic polycyclic aromatic hydrocarbons (PAHs) and petroleum compounds have been observed to be more hydrophobic than other bacteria, allowing them to increase uptake of and promote adhesion to PAHs (9). Although not all hydrophobic bacteria are capable of degrading hydrophobic chemicals (10), those that are exhibit increased biodegradation rates compared to those for less hydrophobic strains as a result of stronger adhesion to non-aqueous-phase liquids (NAPLs) (11, 12). Although the cell surface properties have been used for the separation of bacteria (13–15), these properties might be used more extensively to separate additional organisms of interest.

Organohalide-respiring bacteria are one example of important organisms that can be difficult to enrich and isolate (e.g., see reference 16), and yet they have a great deal of potential in biotechnological applications. Further characterization of the biochemistry and physiology of organohalide-respiring bacteria should facilitate improved bioremediation of hazardous chlorinated chemicals in sediments and groundwater (17). Putative organohalide-respiring bacteria have also been found in uncontaminated environments (18). Although little is known about the biochemistry and physiology of these organisms, they may also have utility in remediation applications.

Because chlorinated compounds are hydrophobic and are often present in the dissolved phase at very low concentrations (18, 19), it is possible that organohalide-respiring bacteria might have developed cell surface characteristics, such as hydrophobic surface moieties, that thermodynamically encourage their adhesion to hydrophobic electron acceptors. *Dehalococcoides mccartyi*, a well-studied organohalide-respiring bacterium, has been found to grow at dense non-aqueous-phase liquid (DNAPL) interfaces (20–22), suggesting that there might be some physiological mechanism by which this interaction occurs. In addition, in uncontaminated environments where concentrations of chlorinated compounds are very low (18, 23), a hydrophobic surface might provide an advantage, promoting the adhesion of organohalide-respiring bacteria to chlorinated electron acceptors. The objective of this research was to test the hypothesis that putative organohalide-respiring bacteria have hydrophobic cell surfaces, facilitating the rapid partial separation of *Dehalococcoides*-like bacteria at an interface between the cell suspension and a hydrophobic liquid. If this hypothesis is correct and can be exploited, it would provide a method for faster separation and improved study of these bacteria.

RESULTS

Separation only. The relative abundance of *Dehalococcoides*-like organisms significantly increased at the interface of a hydrophobic liquid compared to their abundance

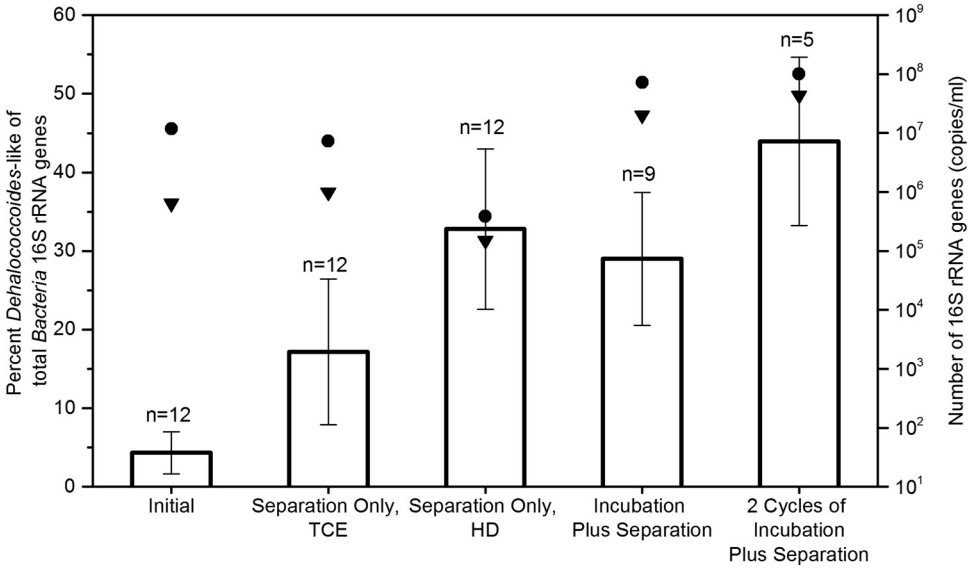

FIG 1 The increases in the percentage of *Dehalococcoides*-like 16S rRNA genes out of the total number of *Bacteria* 16S rRNA genes and the total number of *Dehalococcoides*-like 16S rRNA genes are shown for the separation only method applied to the PCB-enriched sediment-free culture. Open bars represent the percentages of *Dehalococcoides*-like organisms out of the total number of *Bacteria* l6S rRNA genes. Filled triangles (▼) show the quantities of *Dehalococcoides*-like 16S rRNA genes per milliliter of sample, and filled circles (●) show the quantities of total *Bacteria* 16S rRNA genes per milliliter of sample. Error bars represent the 95% confidence intervals. The number above each bar represents the number of replicates of each experiment. The incubation plus separation result is an average of the results for separation after 4, 5, and 6 days.

in initial liquid culture samples. This was true with the polychlorinated biphenyl (PCB)-enriched sediment-free culture and the tetrachloroethene (PCE)-enriched digester sludge when either trichloroethene (TCE) ($P < 0.001$ and $P = 0.02$, respectively) or hexadecane (HD) ($P < 0.001$ for both cultures) was used as the hydrophobic liquid (Fig. 1 and 2, respectively). After only 20 min, the ratios of the 16S rRNA genes of *Dehalococcoides*-like organisms to those of *Bacteria* increased by factors of 4.0 and 8.1 using TCE and HD, respectively, as the NAPL in the PCB-enriched sediment-free culture and by factors of 6.3 and 6.2 using TCE and HD, respectively, as the NAPL in the digester sludge. Because of the short time period associated with these experiments, the interactions of the *Dehalococcoides*-like organisms with the hydrophobic liquid interface must have been a result of physical-chemical interactions rather than metabolic ones (i.e., growth). HD, compared to TCE, increased the relative abundance of

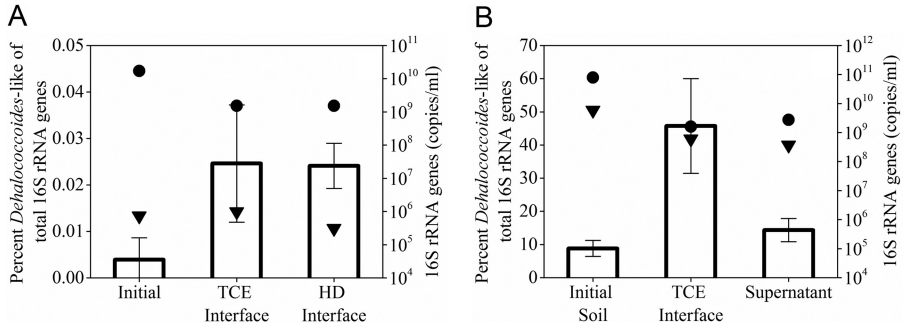

FIG 2 The increases in the percentage of *Dehalococcoides*-like 16S rRNA genes out of the total number of *Bacteria* 16S rRNA genes are shown for the separation only method using both TCE and HD with anaerobic digester sludge (A) and TCE with uncontaminated sediment (B). Filled triangles (▼) show the quantities of *Dehalococcoides*-like 16S rRNA genes per milliliter of sample, and filled circles (●) show the quantities of total *Bacteria* 16S rRNA genes per milliliter of sample. Data for each of the separation methods with the PCB-enriched sediment-free culture are presented. Error bars represent the 95% confidence intervals. Note that the supernatant from the digester sludge experiment was not analyzed.

Dehalococcoides-like bacteria more effectively ($P < 0.005$). No significant differences were observed in the relative abundance of *Dehalococcoides*-like organisms at the interface when the method was performed with larger initial culture volumes (1, 3, and 6 ml of the sediment-free culture) ($P = 0.29$). As expected, the total number of 16S rRNA genes present (*Dehalococcoides*-like and total *Bacteria*) did change in the interface sample with the initial mass used in the separation procedure; therefore, larger initial volumes might be used to obtain a larger overall quantity of 16S rRNA genes if desired. *Dehalogenimonas* and *Dehalobacter* were not detected in the initial PCB-enriched sediment-free culture samples or in six randomly selected interface samples; they were present near the detection limit in the initial and interface samples of the digester sludge. *Desulfitobacterium* was detected at levels near the detection limit in the initial and interface samples of the PCB-enriched sediment-free culture but not in samples taken from the digester sludge experiments. Because of the low levels of or absence of these other putative organohalide-respiring organisms from the two cultures tested, it is uncertain whether this method would result in their partial separation.

The relative abundance of *Dehalococcoides*-like organisms was also significantly ($P = 0.02$) increased at the TCE-reduced anaerobic mineral medium (RAMM) interface when uncontaminated sediment samples were subjected to the separation procedure, increasing from 8.8 $\pm$ 2.4% to 45.7 $\pm$ 16.2% (Fig. 2). These results were supported by Illumina sequencing (see Fig. S2 in the supplemental material), where an increase in the *Chloroflexi* was observed in interface samples. One operational taxonomic unit (OTU), OP8, also appeared to be partially separated at the interface (Fig. S2). The role of this phylum is unknown; nevertheless, OP8 has been observed in high concentrations in hydrocarbon-contaminated soil (24), suggesting that it might also have a niche that benefits from a hydrophobic cell surface. *Dehalogenimonas* and *Desulfitobacterium* organisms were present in the initial and interface samples, but at numbers below the quantification limit of 5×10^2 copies/ml of sample. *Dehalobacter* was not detected in any of the sediment samples.

Mass balances on *Bacteria* and *Dehalococcoides*-like organisms were calculated for several separation experiments. These data are shown in Table S2 in the supporting material. Overall, the numbers of 16S rRNA genes for *Dehalococcoides*-like bacteria and *Bacteria* in the interface samples plus those in the supernatant samples were within about an order of magnitude of those in the initial samples. We considered these mass balances to be reasonable, given that we did not attempt to quantify DNA extraction efficiencies throughout our experiments, which may have been affected by the TCE or HD present in some samples, and quantitative PCR (qPCR) quantification is log normal.

The organisms present in the interface samples from the PCE-enriched digester material were also assayed for dechlorination activity as a way to determine viability after the separation procedure was performed. Dechlorination was tracked by the formation of the daughter product *cis*-dichloroethylene (*cis*-DCE). *cis*-DCE formation was delayed in both the resuspended HD and TCE interface samples (45 days) compared to that observed in the positive control (35 days). This is most likely a result of the smaller amount of biomass in the interface samples compared to that in the positive control and also the fact that there may have been some partitioning of both PCE and daughter products into the small amount of NAPL that remained in the interface samples. *cis*-DCE was formed at maximum observed rates of 15.2, 7.8, and 1.5 μg *cis*-DCE/day in the positive control, HD interface samples, and TCE interface samples, respectively (Fig. 3). The separation procedure did not appear to negatively affect cell activity and viability (see Fig. S4 in the supplemental material). Indeed, the increase in the number of *Dehalococcoides*-like bacteria per mole of chloride released was higher in the TCE interface samples (4.6×10^{13} 16S rRNA gene copies/mol chloride released or 1.4×10^{-7} μg *cis*-DCE produced/16S gene copy/day) compared to that in the positive control (8.9×10^{11} 16S rRNA gene copies/mole chloride released or 7.6×10^{-6} μg *cis*-DCE produced/16S gene copy/day) ($P = 0.004$). No difference was seen in the increase in *Dehalococcoides*-like bacteria per mole of chloride released in the HD

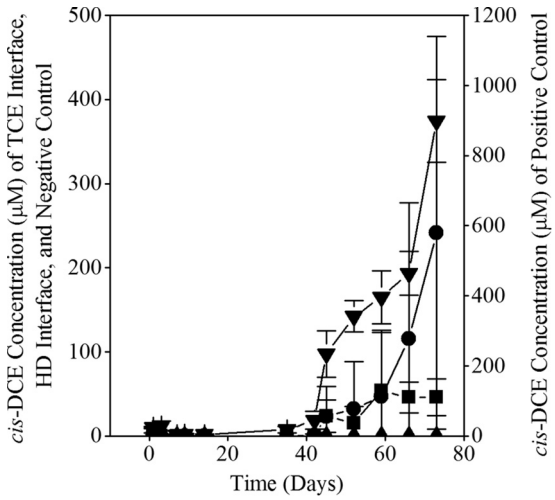

FIG 3 The concentration of *cis*-DCE is shown over time as a result of PCE dechlorination in the sterile negative control (▲), a positive control (▼), resuspended interface samples separated with TCE (■), and resuspended interface samples separated with HD (●). Error bars represent the 95% confidence intervals.

interface samples (8.2×10^{12} 16S rRNA gene copies/mole chloride released or 7.6×10^{-6} μg *cis*-DCE produced/16S gene copy/day) compared to that in the positive control ($P = 0.33$).

Incubation plus separation. When TCE was used as the hydrophobic phase and partial physical separation was combined with incubation, both the number of *Dehalococcoides*-like 16S rRNA genes and their relative abundance were affected (Fig. 1). After a 3-hour incubation period, the total number of *Dehalococcoides*-like 16S rRNA genes increased at the interface from 9.9×10^5 to 1.3×10^7 copies/ml sample, but resulted in a simultaneous decrease in the relative abundance of *Dehalococcoides*-like bacteria, dropping from 17.2% to 9.2%. For longer periods of incubation with TCE followed by the separation procedure, *Dehalococcoides*-like bacteria significantly increased in relative abundance at the interface. For example, after 4 days, the relative abundance of *Dehalococcoides*-like bacteria increased to 34.5 ± 10.31% ($P < 10^{-6}$ compared to the initial culture and $P < 0.001$ compared to separation only with TCE), as did the total number of *Dehalococcoides*-like 16S rRNA genes, from $9.9 \times 10^5 \pm 1.0 \times 10^5$ copes/ml sample after separation only to $2.0 \times 10^7 \pm 1.5 \times 10^6$ copies/ml sample after 4 to 6 days of incubation plus separation (Fig. 1).

This 20.4-fold increase in the number of *Dehalococcoides*-like 16S rRNA genes was observed over a 4-day period, in contrast to only a 9.8-fold increase in total *Bacteria* 16S rRNA genes over the same period. This increase in *Dehalococcoides*-like 16S rRNA genes might be a result of additional partitioning and/or growth at the interface, suggesting that the *Dehalococcoides*-like bacteria were viable and were partially separated as a result of their physical cell properties and also likely were growing at the TCE-medium interface. These results were also supported by Illumina sequencing (see Fig. S3 in the supplemental material), where an increase in the *Chloroflexi* was observed after 1 week of incubation. The percentage of *Dehalococcoides*-like 16S rRNA genes decreased insignificantly in the supernatant, to 4.3%, suggesting that if growth was occurring, it did not occur in the bulk culture medium or growth in the bulk medium was coupled to rapid cell partitioning to the interface. After 4 days, the total number of *Dehalococcoides*-like bacteria remained constant (Fig. 4). This is not surprising, as others have shown that the build up of toxic dechlorination products during enrichment and extended exposure to saturation concentrations of TCE inhibit the growth of *Dehalococcoides mccartyi* strains (25–27).

No detection of *Dehalogenimonas* or *Dehalobacter* occurred during the incubation plus separation experiment. *Desulfitobacterium* organisms were present but were below the quantification limit of 5×10^2 copies/ml sample in all samples.

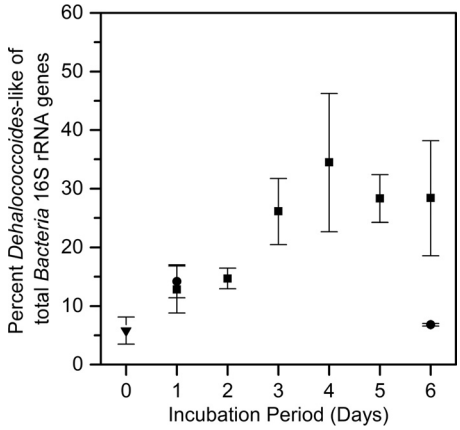

FIG 4 The changes in the percentage of *Dehalococcoides*-like 16S rRNA genes out of the total number of *Bacteria* 16S rRNA genes are shown for the incubation plus separation experiments with the PCB-enriched sediment-free culture. Incubation periods ranged from 1 to 6 days. The initial percentage is shown as a filled triangle (▼), and experiments with HD and TCE are shown with filled circles (●) and filled squares (■), respectively. Error bars represent the 95% confidence intervals.

No increase in the relative abundance (Fig. 4) or the total number of *Dehalococcoides*-like, *Dehalogenimonas*, *Desulfitobacterium*, or *Dehalobacter* 16S rRNA genes was observed when the culture was incubated with HD or HD amended with TCE followed by separation (data not shown). The total number of *Bacteria* 16S rRNA genes did increase by approximately a factor of 3 in those vials.

Cycles of incubation and separation. The intent of cycling incubation and separation steps was to further increase the percentage of and possibly the total number of putative organohalide-respiring bacteria by providing a longer period of incubation at the interface while also removing inhibitors (e.g., dechlorination products) that might build up over time in the vials (25). This was not observed with *Dehalococcoides*-like organisms, as they began to decrease in number after two cycles, and any presumed enrichment of these organisms became very inconsistent. Nevertheless, a consistent increase in total 16S rRNA genes was observed in these experiments, demonstrating that some other organism(s) were apparently enriched. Interestingly, although *Dehalococcoides*-like bacteria did not increase in number or relative abundance through cycles of incubation and separation, species of another putative organohalide-respiring bacterium, *Desulfitobacterium*, did increase in number by more than 3 orders of magnitude (Fig. 5). It is therefore possible that this method might be used to gain insight into additional low-abundance organisms that may play a role in the biodegradation or reduction of hydrophobic organics. Neither *Dehalogenimonas* nor *Dehalobacter* was detected in any of the samples.

DISCUSSION

This study provided evidence that *Dehalococcoides*-like bacteria and *Desulfitobacterium* spp., both of which include putative organohalide-respiring bacteria, are partially separated at the interface between an NAPL and medium and therefore are likely to have a more hydrophobic cell surface than many other organisms. Indeed, the method developed herein to aid in the partial separation of *Dehalococcoides*-like bacteria was based on the assumption that these organisms might possess hydrophobic cell surface properties that would enable them to better attract and respire hydrophobic electron acceptors. A hydrophobic cell surface might provide an evolutionary advantage to *Dehalococcoides*-like and other organohalide-respiring bacteria as has been suggested for a hydrophobic alkane degrader (11, 12, 28). Many chlorinated compounds are sparingly soluble in water, limiting their bioavailability (25). A more hydrophobic cell surface would provide a mechanism for organohalide-respiring bacteria to attach to surfaces where contaminants are sorbed, perhaps enabling better contact between the cell and its hydrophobic electron acceptor.

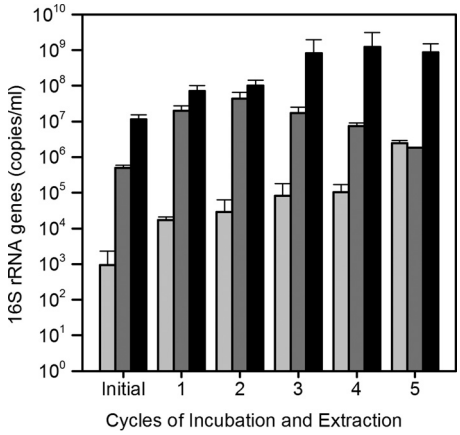

FIG 5 The numbers of 16S rRNA genes (copies per milliliter of sample) of *Desulfitobacterium* (light gray), *Dehalococcoides*-like bacteria (medium gray), and total *Bacteria* (black) are shown from the interface samples after cycles of enrichment and separation with TCE. Error bars represent the 95% confidence intervals.

These results also clearly showed that dechlorinating organisms remained viable after separation (Fig. S4) and that some *Dehalococcoides*-like bacteria and *Desulfitobacterium* spp. appeared to be capable of growth at the medium-hydrophobic liquid interface if the hydrophobic phase was able to serve as an electron acceptor (i.e., TCE). Growth of *Dehalococcoides mccartyi* at an NAPL phase has been suggested by others as well (20–22), further supporting these conclusions.

Two different hydrophobic liquids, TCE and HD, exhibited different separation capabilities, which was likely a result of HD's extreme hydrophobicity (log octanol-water coefficient [log K_{ow}] = 8.2). More hydrophilic cells were potentially excluded from the interface based on their surface free energy (15). This is supported by the lower total number of 16S rRNA genes (both *Dehalococcoides*-like and *Bacteria*) at the interface of HD and the PCB-enriched sediment-free culture compared to the number of 16S rRNA genes at the interface of TCE and the PCB-enriched sediment-free culture (Fig. 1).

The research presented herein provides a simple and rapid method to partially separate *Dehalococcoides*-like organisms from a mixed microbial community, allowing for improved study of *Dehalococcoides*-like bacteria, especially in environmental samples and in previously understudied niches such as uncontaminated lake sediments (18, 29). This is a group of organisms that are thought to be useful from a biotechnological perspective for the bioremediation of chlorinated contaminants and may be important for chloride and carbon cycling in the natural environment (18, 29) but can be difficult to study. Others have developed similar methods to separate cells based on surface properties to characterize a population (e.g., the microbial adhesion to hydrocarbons [MATH] assay), but these are rarely used for cell enrichment and study (10, 14, 15). Indeed, the partial separation of bacteria from one another based on cell surface properties has been confined to a limited number of taxa (15) or to the selection of strains with various properties from a single pure culture (12). This work, therefore, represents a new use of this approach and promises to improve our ability to rapidly identify and separate new organisms of interest. At its simplest, this rapid method for cell separation relies only on the interaction between the cell surface and the interface of a hydrophobic liquid, providing additional opportunities to optimize the method further by manipulating factors that change cell surface characteristics, including pH and ionic strength.

Despite exciting results, this method still has limitations. For example, the incubation and separation procedure is based on the hypothesis that the hydrophobic liquid used can serve as an electron acceptor; without knowledge or availability of preferred electron acceptors, however, this might not be possible. Additionally, as the error bars on the figures indicate, the method can be variable, especially when multiple incuba-

tion and separation steps are used. Nevertheless, multiple individuals performed these experiments, providing validation that, although the method is variable, it is reproducible and effective. Finally, as with most methods designed to partially separate or enrich organisms of interest, this method is not able to isolate organisms of interest and is also not likely to separate all organohalide-respiring bacteria. There are likely to be organohalide-respiring bacteria that do not have a hydrophobic surface and bacteria incapable of organohalide respiration that do. The data presented, however, show that *Dehalococcoides*-like bacteria collected at a much higher fraction than overall *Bacteria* at the interface between an aqueous culture medium and a hydrophobic liquid, supporting the utility of this method for enhancing our knowledge of this important group of bacteria.

MATERIALS AND METHODS

Microbial culture and sediment. The polychlorinated biphenyl (PCB)-enriched sediment-free culture used for separation experiments was originally inoculated with sediment from the Raisin River, Michigan (30) and was enriched over time with 2,3,4,5-tetrachlorobiphenyl, 2,3,4-trichlorobiphenyl, 3,4,5-trichlorobiphenyl, and 3,4-dichlorobiphenyl to produce a sediment-free culture in reduced anaerobic mineral medium (RAMM) (31). Acetate (20 mM) was amended approximately every 4 months. The culture headspace was 4% H_2, which was also replaced approximately every 4 months. The culture was stored in an anaerobic chamber containing 96% N_2 and 4% H_2 (Coy Laboratory Products).

Another culture was enriched from anaerobic digester material obtained from the Empire Wastewater Treatment Plant in Minnesota. In an anaerobic chamber, digester material (5 ml) was diluted into RAMM (65 ml) in six 100-ml serum bottles. The bottles were amended twice with 100 μM tetrachloroethene (PCE) over 2 months to enrich for organisms capable of dechlorination (presumably organohalide-respiring bacteria).

Uncontaminated sediment samples were collected from Pelican Lake, MN (46°36′42.7566″N, 94°9′26.4024″W). Samples were taken at an approximate depth of 0.3 m, placed in sterile bottles, and transported to the laboratory within 24 h for storage in an anaerobic chamber.

Experimental setup. Hydrophobic separation experiments were performed as described below with the PCB-enriched sediment-free culture, the anaerobic digester sludge, or Pelican Lake sediment. All experiments were performed in replicate, as stated below.

(i) Separation only. Separation experiments were performed to determine whether a variety of organohalide-respiring bacteria, including *Dehalococcoides*-like bacteria, in the PCB-enriched sediment-free culture, digester enrichment culture, or uncontaminated sediment possessed a more hydrophobic cell surface than other organisms present in the sample and might therefore be partially separated through mixing with a hydrophobic solvent. For experiments with the PCB-enriched sediment-free culture, an initial sample (1 ml) was taken and centrifuged for microbial analysis (see below). A second aliquot of the culture (3 ml) was placed in a sterile 10-ml glass vial. Approximately 500 μl of a hydrophobic, non-aqueous-phase liquid (NAPL), either hexadecane (HD) (log octanol-water coefficient [log K_{ow}] = 8.2) or trichloroethene (TCE) (log K_{ow} = 2.42), was added to the vial to form a continuous layer of NAPL in contact with the cell culture. TCE is denser than water and therefore formed a layer on the bottom of the vial, whereas HD is less dense and formed a layer on top of the culture (see Fig. S1 in the supplemental material). The vials were shaken vigorously for 30 s and then allowed to stand for 5 min to allow the NAPL and aqueous layer to separate. After this initial mixing period, three cycles of gentle mixing via rolling or swirling the vials for approximately 30 s followed by standing for 3 min were performed. After the last gentle mixing period, the vials were allowed to stand an additional 5 min to allow the NAPL and aqueous layers to completely separate. A 200-μl sample of the cell culture was carefully removed via pipette from the aqueous phase at the interface of the NAPL phase ("interface") for microbial analysis (Fig. S1). In some samples, the cell culture was also sampled (1 ml) at a location away from the interface ("supernatant") for DNA analysis (Fig. S1). When initial, interface, and supernatant samples were obtained, a mass balance over the 16S rRNA gene for *Bacteria* and *Dehalococcoides*-like bacteria was calculated. Both the interface and supernatant samples were centrifuged at 7,000 rpm for 10 min to concentrate the cells present and remove any NAPL that might remain in the sample and interfere with DNA extraction. The experiment was repeated 12 times. In one set of triplicate vials, the HD layer was sampled for microbial analysis.

To verify that the volume of culture used in the separation experiment did not affect the outcome, the experiment was repeated (in triplicate) with volumes of 1, 3, and 6 ml PCB-enriched sediment-free culture, instead of the 3 ml described above, and placed into 3-, 10-, and 20-ml vials, respectively. These experiments were performed identically to those described above except that 0.2, 0.5, and 1 ml of NAPL were added to the vials, respectively.

Experiments were also performed in the same manner with the PCE-enriched digester culture using TCE and HD as the NAPL phase. In these experiments, the viability of the bacteria present in the interface samples after performance of this separation procedure was verified by repeating the experiment and resuspending the interface (approximately 200 μl) in RAMM (10 ml) and amending with 100 μM PCE. The dechlorination of the PCE and formation of the daughter products TCE and *cis*-dichloroethylene (*cis*-DCE) were then monitored with time by gas chromatography (GC). A positive control (1 ml of enrichment culture in 9 ml of RAMM) and a negative control (10 ml of sterile RAMM) were also amended with 100

μM PCE and monitored for PCE dechlorination and daughter product formation. Experiments were performed in quadruplicate in the anaerobic chamber.

For experiments with Pelican Lake sediment, bacteria were removed from the sediment prior to subjecting them to the separation procedure. Briefly, 5 ml of sediment and 5 ml of RAMM were mixed in a blender (Waring commercial) for 3 min on the low speed setting. Soil particles were allowed to settle for 5 min, and the liquid fraction was removed. The separation method as described above for the PCB-enriched sediment-free cell culture was performed with 3 ml of this liquid fraction. The DNA from these samples was processed using the bead-beating method, as described below; therefore, the Pelican Lake samples were not centrifuged prior to DNA extraction. Only TCE was tested as the NAPL phase in experiments with sediment. The experiment was performed in triplicate.

(ii) Incubation plus separation. Experiments were also performed in which the PCB-enriched sediment-free culture was left in contact with the NAPL phase (TCE, HD, or HD containing 200 μM TCE [HD+TCE]) for up to 1 week prior to sampling and analyzing the interface. The culture contained 20 mM acetate, H_2 (4%) in the headspace, and titanium nitrilotriacetic acid (100 μM, final concentration) (32) to maintain reduced conditions in the vials, based on the color indicator resazurin (1 mg/ml).

The procedure was performed as described above for the separation only experiments, except that instead of removing the interface for analysis after 20 min, the 10-ml vials were crimped closed in the anaerobic chamber and placed on a rotator at 20 rpm for various periods of time. All vials were started with a common initial culture that was also sampled for microbial analysis. At specified time points, triplicate vials were removed from the rotator and allowed to stand for 5 min before they were sacrificed for sampling of the interface and supernatant as described above. Experiments with HD or HD+TCE as the NAPL phase were sampled after 1 and 7 days of incubation. Experiments with TCE as the NAPL phase were sampled after 1, 2, 3, 4, 5, 6, and 7 days of incubation.

Cycles of incubation and separation. Multiple incubation and separation cycles were also tested to determine whether further concentration and enrichment of putative organohalide-respiring bacteria might be achieved if multiple partial separation events were combined with incubation. This procedure allowed for a longer incubation period but also prevented the build up of by-products by providing fresh medium to the organisms present. The vials were prepared as described in the incubation plus separation experiments above. Only TCE was used as the hydrophobic phase for these experiments because it is a possible electron acceptor for some *Dehalococcoides*-like bacteria. Three milliliters of PCB-enriched sediment-free culture (containing 20 mM acetate) was mixed with 500 μl of TCE in a crimp top vial (10 ml) in an anaerobic chamber. The headspace of the vials contained 4% H_2. As in the week-long experiment, the total numbers of vials required for sacrificial analysis throughout the experiment were started with the same initial culture. The vials were mixed on a rotator in the anaerobic chamber at 20 rpm for 4 days. After the initial incubation, one set of triplicate vials underwent the separation procedure (described above), and the interface was analyzed. The remaining four sets of triplicate vials were also subjected to the separation procedure, performed in the anaerobic chamber, and each interface sample (200 μl) was transferred to a 10-ml vial containing 3 ml of sterile RAMM and 500 μl of fresh TCE. Mixing of the vials was then continued on a rotator in the anaerobic chamber for another 4 days. This 4-day incubation cycle followed by a separation step was performed 1 to 5 times, with all vials sacrificed for microbial analysis or transferred in triplicate.

Microbial analysis. DNA from the interface, supernatant, and initial culture samples was extracted for further analysis. Samples from experiments performed with the PCB-enriched sediment-free culture or digester sludge were extracted using the FastDNA extraction kit (MP Bio); samples from experiments performed with sediment were extracted and purified using a soil DNA extraction kit (MoBio). The HD phase was also extracted from triplicate vials in one preliminary experiment, but no DNA was detected (data not shown); therefore, subsequent sampling of the hydrophobic phases was not continued.

Previously developed qPCR primers were used to quantify the relative number of *Dehalococcoides*-like bacteria (18), *Dehalogenimonas* (33), *Desulfitobacterium* (34), and *Dehalobacter* (34) in the samples. The *Dehalococcoides*-like primers target bacteria that are phylogenetically similar to *Dehalococcoides mccartyi* but also include *Dehalogenimonas* spp. and a broader group of the *Chloroflexi*, including organisms found in uncontaminated environments (18). The organisms targeted by these primers are almost certainly not all organohalide-respiring bacteria but contain known organohalide-respiring bacteria and target phylogenetically related unknown and less-studied bacteria that may in fact be organohalide respiring (35, 36). The total number of 16S rRNA genes was quantified with general bacterial primers targeting the V3 region of the 16S rRNA gene (37). The qPCR mixture (15 μl) contained 1× SYBR green master mix (Bio-Rad Laboratories), 100 nM each primer, 1 mg/liter of bovine serum albumin (BSA), and 1 μl of undiluted template. The general qPCR cycle was a 95°C initial denaturation for 10 min followed by 40 cycles of 95°C denaturation for 15 s and 1-min anneal/extension at the specific annealing temperature for each primer set. A melting curve analysis was completed at the end of each run for quality control/assurance. Additionally, DNA from an interface, supernatant, and initial culture sample was diluted (1:5, 1:10, and 1:100) and quantified to ensure that PCR inhibitors did not affect quantification; there was no evidence of PCR inhibitors in the undiluted template. The number of gene copies in each sample was determined with a standard curve of 10-fold dilution standards ranging from 10^9 to 10^2 for total *Bacteria* 16S rRNA genes and 10^8 to 10^0 for all other target organisms. Standards were made by ligating the 16S rRNA gene from the target organism into pGEM-T Easy vectors (Promega) according to the manufacturer's instructions. This was transformed into *Escherichia coli* JM109. Plasmids were purified using a MiniPrep kit (Qiagen). Hoechst 33258 dye was used to stain the DNA for quantification on a TD-700 fluorometer using calf thymus as the DNA standard. Limits of quantification for *Dehalococcoides*-like bacteria, *Dehalogenimonas*, *Desulfitobacterium*, *Dehalobacter*, and total *Bacteria*

16S rRNA genes with these methods were 1×10^3, 5×10^2, 5×10^2, 5×10^2, and 5×10^4 copies/ml of sample, respectively.

Illumina sequencing was also performed to determine whether specific organisms were partially selected for or against at the HD or TCE interface. Briefly, the microbial communities of the initial culture, interface, and supernatant were compared using Illumina sequencing technology. The same primer sequences used to quantify the total number of 16S rRNA genes were used to sequence the V3 region of the 16S rRNA genes (37). Additionally, the primers contained the sequences required to bind to the Illumina platform, a 6-bp tag for multiplexing, and a random sequence ranging from 1 to 6 bp to limit PCR bias due to the different tags (38). PCR was used to amplify the 16S rRNA gene fragment following the method outlined in Bartram et al. (38). Illumina MiSeq paired-end sequencing (2×150) was completed by the University of Minnesota Genomics Center (UMGC). Analysis of the sequencing reads was completed with Quantitative Insights into Microbial Ecology (QIIME) through the Minnesota Supercomputing Institute (MSI) (39). In summary, reads were trimmed to 125 bp before the forward and reverse reads were paired. All sequences with a *Q* score of less than 35 were removed. *De novo* OTU picking was performed using the UCLUST algorithm (40). Taxonomy was assigned to each OTU with the RDP Classifier based on the most abundant sequence in the OTU (41). Spurious Illumina results were observed when the initial quantity of 16S rRNA gene copies was less than about 5×10^7 total copies/ml of sample and the initial quantity of *Dehalococcoides*-like 16S rRNA gene copies was less than about 5×10^6 copies/ml of sample. This was thought to be a result of background signal (42, 43) or PCR bias (38, 44, 45).

Chemical analysis. Headspace samples (20 μl) were taken to quantify PCE, TCE, 1,1-DCE, *cis*-DCE, and *trans*-DCE in vials by GC with an electron capture detector. The oven temperature was maintained at 40°C, the inlet at 300°C, and the detector at 325°C. The nitrogen gas flow rate was 2 ml/min. Method detection limits were $<$100 nM for PCE and TCE and 5 μM for 1,1-DCE, *cis*-DCE, and *trans*-DCE. Vinyl chloride and ethene were not quantified.

Data analysis. The fraction or relative abundance of *Dehalococcoides*-like 16S rRNA genes was defined as the quantity of *Dehalococcoides*-like 16S rRNA genes divided by the quantity of total *Bacteria* 16S rRNA genes. This is generally expressed as the percentage of *Dehalococcoides*-like 16S rRNA genes out of the number of quantified *Bacteria* 16S rRNA genes. Two-tailed Student's *t* tests were performed with Microsoft Excel to determine significance. A *P* value of <0.05 was considered significant.

Accession number(s). The raw sequences from the amplicon libraries were deposited in the Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR4436127 to SRR4436134.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02946-16.

TEXT S1, PDF file, 1.4 MB.

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