Dehalogenimonas alkenigignens sp. nov., a chlorinated-alkane-dehalogenating bacterium isolated from groundwater

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Two strictly anaerobic bacterial strains, designated IP3-3T and SBP-1, were isolated from groundwater contaminated by chlorinated alkanes and alkenes at a Superfund Site located near Baton Rouge, Louisiana (USA). Both strains reductively dehalogenate a variety of polychlorinated aliphatic alkanes, including 1,2-dichloroethane, 1,2-dichloropropane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane and 1,2,3-trichloropropane, when provided with hydrogen as the electron donor. To clarify their taxonomic position, strains IP3-3T and SBP-1 were characterized using a polyphasic approach. Both IP3-3T and SBP-1 are mesophilic, non-spore-forming, non-motile and Gram-stain-negative. Cells of both strains are irregular cocci with diameters of 0.4–1.1 μm. Both are resistant to ampicillin and vancomycin. The genomic DNA G+C contents of strains IP3-3T and SBP-1 are 55.5±0.4 and 56.2±0.2 mol% (HPLC), respectively. Major cellular fatty acids include C18:1ω9c, C16:0, C14:0 and C16:1ω9c. 16S rRNA gene sequence based phylogenetic analyses indicated that the strains cluster within the phylum Chloroflexi most closely related to but distinct from the species Dehalogenimonas lykanthroporepellens (96.2% pairwise similarity) and Dehalococcoides mccartyi (90.6% pairwise similarity). Physiological and chemotaxonomic traits as well as phylogenetic analysis support the conclusion that these strains represent a novel species within the genus Dehalogenimons for which the name Dehalogenimons alkenigignens sp. nov. is proposed. The type strain is IP3-3T (=JCM 17062T =NRRL B-59545T).

At present, the genus Dehalogenimons contains a single species, Dehalogenimons lykanthroporepellens, the type strain of which was isolated from groundwater containing high concentrations of chlorinated solvents (Moe et al., 2009). Strains of this strictly anaerobic species are able to reductively dehalogenate a variety of industrially and environmentally important polychlorinated ethanes and propanes. Due to spills and past inappropriate disposal methods, these chlorinated organic compounds are prevalent contaminants of groundwater and soil (De Wildeman & Verstraete, 2003; Fletcher et al., 2009). PCR-based methods to detect and quantify Dehalogenimons lykanthroporepellens indicated that the species had relatively wide spatial distribution and high cell numbers in the groundwater at the site from which it was first isolated (Yan et al., 2009b), even with the presence of dense non-aqueous-phase liquids in the subsurface (Bowman et al., 2006).

To characterize the microbial population present in groundwater of the Scenic Highway portion of the PetroProcessors of Louisiana, Inc. (PPI) Superfund site located near Baton Rouge, LA (USA), 454 pyrosequencing was used to construct 16S rRNA gene libraries from groundwater collected from various wells between January and December 2009. Analysis of the gene libraries revealed that groundwater from two wells contained a large
percentage of sequences (7.7 %, 4617 of 60 334 sequences, and 9.3 %, 3478 of 37 583 sequences) clustering within the class *Dehalococcoidia* (Loeffler et al., 2013) of the phylum *Chloroflexi* but with <97 % sequence identity with previously isolated strains with sequences in the public databases. A subsequent effort to isolate representatives of these bacterial groups resulted in recovery of two novel anaerobic bacterial strains, IP3-3T and SBP-1, which were characterized using a polyphasic approach to clarify their taxonomic position.

Groundwater was collected from PPI Superfund site monitoring wells with locations, contaminant concentrations and geochemistry as summarized in Tables S1 to S3 available in IJSEM Online. Immediately following collection (in sterile glass bottles filled leaving little or no gas headspace), the groundwater was amended with titanium citrate solution (Zehnder & Wuhrmann, 1976) to a final concentration of 1 mM Ti(III) and 2 mM citrate to maintain anaerobic conditions during transport to the laboratory.

An enrichment culture was established from groundwater collected from well ID no. IP-3 by inoculating 10 ml groundwater into a 160 ml serum bottle containing 90 ml defined basal medium prepared as described by Yan et al. (2009a) except that titanium citrate [1 mM Ti(III) and 2 mM citrate] replaced sodium sulfide as a reducing agent and 5 mM acetate was replaced by 5 mM each of acetate, pyruvate and lactate. The medium was amended with 1,1,2-trichloroethane (1,1,2-TCA) to reach a final aqueous-phase concentration of 0.5 mM. After four months incubation under dark, static conditions at 30 °C, the enrichment culture was diluted to 10^-10 using 1,1,2-TCA spiked media as prepared for the enrichment culture. After incubation at 30 °C in the dark for two months, all of the 1,1,2-TCA was transformed to vinyl chloride. Following seven consecutive transfers of the culture [with 2 % (v/v) inoculum] and repeated dilution-to-extinction, strain IP3-3T was recovered from a 10^-10 dilution. Strain SBP-1 was recovered using an identical dilution-to-extinction approach but with groundwater collected from well ID no. SBP-017-B. The strains were preserved in anaerobic medium amended with 5 % (v/v) filter-sterilized DMSO and stored at -80 °C.

Culture purity was confirmed by microscopy and observation of single bands in denaturing gradient gel electrophoresis (DGGE) following extraction of genomic DNA and PCR amplification using universal bacterial primers 341f and 907r as described previously (Li & Moe, 2004). PCR employing primers DHC 774 and DHC 1212 (Hendrickson et al., 2002) and primers 582f and 728r (Duhamel et al., 2004) targeting 16S rRNA gene sequences unique to *Dehalococcoides* strains did not produce amplicons. PCR employing primers BL-DC-727f and BL-DC-1020r (Yan et al., 2009b) targeting 16S rRNA gene sequences unique to *Dehalogenimonas lykanthroporepellens* also did not produce amplicons. Unless stated otherwise, all tests were carried out following strict anaerobic procedures in basal medium prepared as described and used in the isolation of strain IP3-3T except that acetate, pyruvate and lactate concentrations were lowered from 5 mM to 0.05 mM. Experiments were performed in duplicate, with H_{2} : CO_{2} : N_{2} (10 % : 10 % : 80 %, by vol.) as the headspace gas, a 2 % (v/v) inoculum and incubation in the dark at 30 °C without shaking. Chlorinated solvent concentrations and degradation products in the headspace gas and the aqueous phase were quantified by gas chromatography as described by Yan et al. (2009a). *Dehalogenimonas lykanthroporepellens* BL-DC-9T was included in all testing for comparative purposes.

The potential of strains IP3-3T and SBP-1 to reductively dehalogenate various chlorinated alkanes [1,2,3-trichloropropane (1,2,3-TCP), 1,2-dichloropropane (1,2-DCP), 1-chloropropane, 2-chloropropane, 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA), 1,1,1-trichloroethane, 1,1,2-trichloroethane (1,1,2-TCA), 1,2-dichloroethane (1,2-DCA), 1,1-dichloroethane, tetrachloromethane (carbon tetrachloride), trichloromethane (chloroform) and dichloromethane (methylene chloride)], chlorinated alkenes (perchloroethene, trichloroethene, cis-1,2-dichloroethene, trans-1,2-dichloroethene, vinyl chloride), chlorinated benzenes (1,2-dichlorobenzene, chlorobenzene) was determined in serum bottles (25 ml) each containing 15 ml basal medium. Chlorinated solvents were purchased from Sigma–Aldrich except trichloroethene (Mallinckrodt Baker), chloroform (Fisher Scientific) and dichloromethane (Fisher Scientific). Other than 1,1,2-TCA (96 % purity), chloroform [American Chemical Society (ACS) reagent grade] and dichloromethane (pesticide grade), all chemicals used were ≥98 % purity. Certified gas standards for vinyl chloride (1002 p.p.m. in N_{2}), ethene (99.5 %) and propane (99 %) were purchased from Sigma–Aldrich. Neat filter-sterilized chlorinated solvents were added to reach approximately 0.5 mM aqueous phase concentration after equilibration, except for vinyl chloride which was supplied via the gas phase to reach a final aqueous phase concentration of 0.03 mM after equilibration. Inoculated serum bottles spiked with 1,1,2-TCA served as positive controls, and uninoculated, chlorinated-solvent-spiked bottles served as negative controls to account for abiotic transformation. After two months incubation, headspace gas and aqueous phases were analysed for chlorinated solvents and potential degradation products by gas chromatography (Yan et al., 2009a).

Strains IP3-3T, SBP-1 and *Dehalogenimonas lykanthroporepellens* BL-DC-9T all reductively dehalogenated 1,2-DCA to ethene, 1,2-DCP to propane, 1,1,2-TCA to vinyl chloride and 1,1,2,2-TeCA to a mixture of cis- and trans-dichloroethene. In the titanium-citrate-reduced medium tested in this study, all three of the strains also reductively dehalogenated 1,2,3-TCP with the production of allyl chloride (3-chloro-1-propene) as an intermediate product. As observed previously (Yan et al., 2009a), allyl chloride is
an unstable compound and underwent hydrolysis to form allyl alcohol. In this study, allyl chloride and allyl alcohol were the only volatile products detected from 1,2,3-TCP dechlorination. Other products reported by Yan et al. (2009a) produced from abiotic reactions of allyl chloride with sulfide (e.g. diallyl sulfide, diallyl disulfide) were not detected. This is consistent with the fact that sulfide was not used as a reducing agent in the present study. Neither IP3-3T, SBP-1 or Dehalogenimonas lykanthroporepellens BL-DC-9T utilized the other chlorinated compounds tested as potential electron acceptors.

To assess whether reductive dechlorination was linked with cell growth, direct cell counts were performed using a DMRXAZ microscope (Leica) equipped with an A6 filter set (360/40 nm excitation, 470/40 nm emission; Leica) and Slidebook 4.1 imaging software (Intelligent Imaging Innovations) after fixing with glutaraldehyde, staining with 4′,6-diamidino-2-phenylindole (DAPI) and collection on 0.2 μm filters (Supor-200, Pall). In bottles amended with 1,2-DCA, 1,2-DCP, 1,2-TCA, 1,1,2,2-TeCA, cell concentrations of strains IP3-3T and SBP-1 determined after dechlorination was complete ranged from 22 to 52 times higher than those of controls prepared and incubated under identical concentrations but lacking chlorinated solvents. For 1,2,3-TCP, cell concentrations of strains IP3-3T and SBP-1 determined after dechlorination was complete ranged from five to eight times higher than controls prepared and incubated under identical concentrations but lacking chlorinated solvents. The ability of strains IP3-3T and SBP-1 to use acetate (5 mM), butyrate (5 mM), citrate (5 mM), ethanol (5 mM), formate (5 mM), fructose (5 mM), fumarate (5 mM), glucose (5 mM), lactate (5 mM), lactose (5 mM), succinate (5 mM), yeast extract (0.5 g l⁻¹) as potential electron donors for reductive dechlorination was assessed in 25 ml serum bottles containing 15 ml basal media. Substrates were added from filter-stereilized 100 × 10 × 25 mm stock solutions. Separate replicates were prepared with headspace gas comprised of CO₂ : N₂ (5% : 95%, by vol.) and H₂ : CO₂ : N₂ (10% : 10% : 80%, by vol.). Filter-sterilized 1,1,2-TCA (neat) was added to reach a final aqueous-phase concentration of 0.5 mM. Bottles were incubated for two months prior to determination of the concentrations of chlorinated solvent and degradation products.

In the absence of H₂ in the gas headspace, neither IP3-3T nor SBP-1 dechlorinated 1,1,2-TCA when supplied with any of the potential electron donors tested. In the presence of H₂ supplied in the gas headspace, 1,1,2-TCA dechlorination to vinyl chloride was essentially complete for all compounds tested except for propionate and lactate.

The temperature and pH range for IP3-3T and SBP-1 to reductively dechlorinate 1,2-DCP was determined in 25 ml serum bottles containing 15 ml anaerobic basal medium amended with 0.5 mM 1,2-DCP and 5 mM acetate in place of 0.05 mM each of acetate, pyruvate and lactate. Temperatures tested were 10, 18, 23.5, 30, 34, 37, 42 and 45 °C. For pH tests, media was adjusted with 1 M NaOH or 2 M HCl to reach pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. Concentrations of 1,2-DCP and the degradation product propene were measured after two weeks and two months incubation. Samples in which more than 5% of the chlorinated solvent was transformed were scored as positive for dechlorination. Both IP3-3T and SBP-1 reductively dechlorinated 1,2-DCP in the temperature range from 18–42 °C (optimum 30–34 °C) but not at temperatures ≤10 °C or ≥45 °C. Reductive dechlorination occurred in the pH range of 6.0–8.0 (optimum pH 6.5–7.5), but not at pH ≤5.5 or ≥8.5.

The ability of IP3-3T, SBP-1 and Dehalogenimonas lykanthroporepellens BL-DC-9T to reductively dechlorinate 1,2-DCP at various salt concentrations was determined as described above but in media supplemented with NaCl at concentrations of 1, 2 and 3% (w/v). Strains IP3-3T and SBP-1 dechlorinated at NaCl concentrations of 1% (w/v), but not at 2%. In contrast, Dehalogenimonas lykanthroporepellens BL-DC-9T dechlorinated 1,2-DCP in media containing NaCl at a concentration of 2% (w/v).

The ability of strains IP3-3T, SBP-1 and Dehalogenimonas lykanthroporepellens BL-DC-9T to grow in the presence of oxygen was tested as described by Yan et al. (2009a). In anaerobically prepared positive controls, reductive dechlorination was complete after two weeks incubation. After two months incubation, no dechlorination occurred in oxygen-purged serum bottles.

To assess the ability to form visible colonies on solid media, strains IP3-3T, SBP-1 and Dehalogenimonas lykanthroporepellens BL-DC-9T were streaked on 65 ml basal media solidified with agar (15 g l⁻¹) in a 160 ml serum bottle, amended with 0.5 mM 1,2-DCP. No colonies were observed even after two months incubation for any of the strains.

The ability of the strains to reductively dechlorinate in medium containing sulfide rather than titanium citrate as the reducing agent was determined in 25 ml serum bottles containing 15 ml anaerobic basal medium amended with 0.5 mM 1,2-DCP and either 1.5 or 3 mM sodium sulfide with incubation for two months. Identically prepared medium containing titanium citrate served as a positive control. All three strains dechlorinated 1,2-DCP in titanium-citrate-reduced medium and medium containing 1.5 mM sulfide, but only Dehalogenimonas lykanthroporepellens BL-DC-9T dechlorinated 1,2-DCP in medium containing 3 mM sulfide.

Cells of strains IP3-3T, SBP-1 and Dehalogenimonas lykanthroporepellens BL-DC-9T grown on 1,2-DCP were Gram stained and visualized by light and scanning electron microscopy (SEM). Motility was assessed using phase-contrast light microscopy (Microphot-FXA; Nikon). Cell morphology was determined from SEM in media.
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The genomic DNA G+C content of strains IP3-3T and SBP-1 was determined by HPLC as described by Mesbah et al. (1989) following DNA isolation using an UltraClean Water DNA Isolation kit (MoBio Laboratories). The G+C content of genomic DNA of strains IP3-3T and SBP-1 as determined by HPLC was 55.5 ± 0.4 mol% and 56.2 ± 0.2 mol%, respectively.

Cells for analysis of cellular fatty acids were grown in anaerobic basal medium prepared as described by Moe et al. (2009) with titanium citrate solution (Zehnder & Wuhrmann, 1976) as a reducing agent at a final concentration of 1.0 mM Ti(III), 2.0 mM citrate. The medium was supplemented with 0.5 mM 1,1,2-trichloroethane and 5 mM sodium acetate (in place of 0.05 mM each of acetate, pyruvate and lactate). Cells were harvested via centrifugation (10 000 g) during the mid-exponential growth phase following incubation for three weeks. Cellular fatty acids were extracted, saponified and methylated according to the protocol of the Sherlock v. 6.0 Microbial Identification System (MIDI). The fatty acids were analysed by a GC equipped with the Microbial Identification software package (Sasser, 1990) with peak identification using the MOORE 6.0 and TSBA 6.0 libraries. The major cellular fatty acids of both strains IP3-3T and SBP-1 included C18:1ω9c, C16:0, C14:0 and C16:1ω9c (Table 1).

Based on 16S rRNA gene sequence comparisons, strains IP3-3T and SBP-1 cluster with Dehalogenimonas

![Fig. 1. Neighbour-joining dendrogram based on analysis of 16S rRNA gene sequences showing the phylogenetic relationship of strain IP3-3T and SBP-1 to taxa of the phylum Chloroflexi. Bootstrap values expressed as a percentage of 1000 resamplings are shown at branch points with significant support. Bar, 2 substitutions per 100 nt positions.](http://ijs.sgmjournals.org)
lykanthroporepellens BL-DC-8 and BL-DC-9T but represent a distinct lineage (96.0 % to 96.2 % sequence similarity) supported by a high bootstrap value (Fig. 1), within the recently described order Dehalococcoidales in the class Dehalococcoidia of the phylum Chloroflexi (Löffler et al., 2013). The low sequence similarity (~96 %) in 16S rRNA gene sequences of strains IP3-3T and SBP-1 when compared with Dehalogenimonas lykanthroporepellens in combination with phenotypic differences supports classification as a different species.

Additional genomic and chemotaxonomic characteristics differentiate IP3-3T and SBP-1 from Dehalogenimonas lykanthroporepellens strain BL-DC-9T. The genomic DNA G+C contents of strains IP3-3T and SBP-1 as determined by HPLC (55.5 ± 0.4 and 56.2 ± 0.2, respectively) are slightly higher than those of Dehalogenimonas lykanthroporepellens strains BL-DC-8 and BL-DC-9T (54.0 and 53.8 mol%, respectively, Moe et al., 2009) determined using the same method.

The major cellular fatty acids determined for strains IP3-3T and SBP-1 (C16:1ω9c, C16:0, C14:0 and C16:1ω9c) were also present in Dehalogenimonas lykanthroporepellens BL-DC-8 and BL-DC-9T. The proportion of C16:1ω9c, however, was higher in strains BL-DC-8 and BL-DC-9T and the proportion of C14:0 was higher in IP3-3T and SBP-1 (Table 1). The proportion of unknown 11.980 reached moderate levels in strains IP3-3T and SBP-1 (7.0 and 4.5 %, respectively) but was undetectable in Dehalogenimonas lykanthroporepellens strains. Summed feature 5 (C18:2ω6c, C18:1ω9c and/or C18:0 anteiso) comprised 6.3 % and 7.7 % in Dehalogenimonas lykanthroporepellens strains BL-DC-9T and BL-DC-8 but was undetected in IP3-3T and was present in only trace amounts (0.8 %) in SBP-1. Unidentified cellular fatty acids with equivalent chain-lengths (ECLs) of 11.980, 13.937 and 15.056, as well as C18:3ω6c(6,9,12), were also detected in strains IP3-3T and SBP-1 but not in Dehalogenimonas lykanthroporepellens strains (Table 1). These differences in fatty acid composition clearly distinguish IP3-3T and SBP-1 as a separate species from Dehalogenimonas lykanthroporepellens.

Strains IP3-3T and SBP-1 share several common phenotypic features with Dehalogenimonas lykanthroporepellens strains BL-DC-8 and BL-DC-9T including strictly anaerobic respiration, requirement for H2 as an electron donor, use of polyhalogenated alkanes as electron acceptors, coupling of cell growth with reductive dehalogenation, Gram-negative staining, small irregular cell morphology and resistance to the antibiotics ampicillin and vancomycin (Moe et al., 2009). As with Dehalogenimonas lykanthroporepellens BL-DC-9T and BL-DC-8, all reductive dechlorination reactions determined to date for strains IP3-3T and SBP-1 appear to involve an exclusively dihaloelimination reaction mechanism (involving simultaneous removal of two chlorines from adjacent carbon atoms and formation of a carbon–carbon double bond). However, IP3-3T and SBP-1 differ from Dehalogenimonas lykanthroporepellens strain BL-DC-9T phenotypically in their inability to reductively dehalogenate in the presence.

### Table 1. Comparison of the cellular fatty acids of strains IP3-3T and SBP-1 (this study) with Dehalogenimonas lykanthroporepellens strains BL-DC-9T and BL-DC-8 (Moe et al., 2009)

Fatty acids occurring at less than 1 % in all strains are not listed. –, Not detected.

<table>
<thead>
<tr>
<th>PLFA</th>
<th>ECL</th>
<th>Amount of PLFA (% of total)</th>
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</thead>
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<tr>
<td></td>
<td></td>
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</tr>
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<td>11.980</td>
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<tr>
<td>C12:0</td>
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<td>Summed feature 8‡</td>
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*Summed feature 3: C16:1ω7c and/or C16:1ω6c and/or C16:0 is 2-OH.
†Summed feature 5: C18:2ω6c and/or C18:2ω9c and/or C18:0 anteiso.
‡Summed feature 8: C18:1ω7c and/or C18:1ω6c.
of 2% NaCl or in medium containing 3 mM sodium sulfide.

On the basis of genotypic, chemotaxonomic and phenotypic features, the newly isolated strains IP3-3T and SBP-1 are clearly distinct from other species in the phylum Chloroflexi. We propose that strains IP3-3T and SBP-1 be placed as a new species within the genus Dehalogenimonas with the name Dehalogenimonas alkenigignens sp. nov.

**Description of Dehalogenimonas alkenigignens sp. nov.**

Dehalogenimonas alkenigignens (al.ke.ni.gi.gnens. N.L. n. alkenum alkene; L. part. adj. gignens giving birth to, producing; N.L. part. adj. alkenigignens producing alkene, because alkene are produced during anaerobic reductive dehalogenation of chlorinated alkanes).

Cells are Gram-negative staining, non-motile, non-spore-forming, irregular cocci with diameter of 0.4–1.1 μm. Reductively dehalogenates polychlorinated aliphatic alkanes including 1,2-dichloroethane, 1,2-dichloropropane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane and 1,2,3-trichloropropane. Chlorobenzene, chloroform, 1-chloropropane, 2-chloropropane, 1,2-dichlorobenzene, cis,1,2-dichloroethene, trans,1,2-dichloroethene, 1,1-dichloroethane, dichloromethane, tetrachloroethene, tetradichloroethane, 1,1,1-trichloroethane, trichloroethene, or vinyl chloride do not serve as electron acceptors. Allyl chloride is produced when grown with 1,2,3-trichloropropane as an electron acceptor and titanium citrate as a reducing agent. Utilizes H2 as an electron donor. Dechlorination is not supported by acetate, butyrate, citrate, formate, fumarate, lactate, propionate, pyruvate, succinate, ethanol, methanol, fructose, glucose, lactose, methyl ethyl ketone, or yeast extract in the absence of H2. Resistant to ampicillin (1.0 g l−1) and vancomycin (0.1 g l−1). Grows at 18–42 °C (optimum 30–34 °C) and pH 6.0–8.0 (optimum pH 6.5–7.5). Growth does not occur at NaCl concentrations ≥2% (w/v). Does not dechlorinate in 3 mM sulphide-reduced media. Major cellular fatty acids include C18:1ω9c, C16:0, C14:0 and C16:1ω9c.

The type strain, IP3-3T (=JCM 17062T=NRRL B-59545T), was isolated from chlorinated-solvent-contaminated groundwater at the PPI, Superfund Site, located near Baton Rouge, LA (USA). SBP-1, isolated from groundwater at the same site, is a second strain of the species. The G+C content of genomic DNA of the type strain as determined by HPLC is 55.5 ± 0.4 mol%.

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**References**


