

Dehalogenimonas lykanthroporepellens gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater

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Two recently reported bacterial strains that are able to reductively dehalogenate polychlorinated aliphatic alkanes, including 1,2,3-trichloropropane, 1,2-dichloropropane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane and 1,2-dichloroethane, were further characterized to clarify their taxonomic position. The two strains, designated BL-DC-8 and BL-DC-9^T, were mesophilic, non-spore-forming, non-motile, Gram-negative staining and strictly anaerobic. Cells were irregular cocci, 0.3–0.6 µm in diameter. The two strains were resistant to ampicillin and vancomycin. Hydrogen was utilized as an electron donor. The genomic DNA G+C content of strains BL-DC-8 and BL-DC-9^T was 54.0 and 53.8 mol%, respectively. The major cellular fatty acids were C_{18:1ω9c}, C_{16:1ω9c}, C_{16:0} and C_{14:0}. Phylogenetic analyses based on 16S rRNA gene sequences indicated that the strains cluster within the phylum *Chloroflexi*, but are related only distantly to all recognized taxa in the phylum. Morphological, physiological and chemotaxonomic traits as well as phylogenetic analysis support the conclusion that these two strains represent a novel species of a new genus in the phylum *Chloroflexi*, for which the name *Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov. is proposed. The type strain of *Dehalogenimonas lykanthroporepellens* is BL-DC-9^T (=ATCC BAA-1523^T =JCM 15061^T).

The phylum *Chloroflexi* is a deep branching lineage within the domain *Bacteria*. The phylum contains at least six major lineages, five of which, at the time of writing, have cultured representatives. These lineages include the classes *Anaerolinea* (Yamada *et al.*, 2006), *Caldilinea* (Yamada *et al.*, 2006), '*Chloroflexi*' (Castenholz, 2001) and *Thermomicrobia* (Hugenholtz & Stackebrandt, 2004). Also included in the phylum *Chloroflexi* is a lineage for which the class '*Dehalococcoidetes*' was informally proposed by Hugenholtz & Stackebrandt (2004) to accommodate the

tetrachloroethene-respiring coccus '*Dehalococcoides ethenogenes*' (Maymó-Gatell *et al.*, 1997).

Two novel anaerobic bacterial strains that cluster within the phylum *Chloroflexi*, designated BL-DC-8 and BL-DC-9^T, were recently demonstrated to be able to reductively dehalogenate a variety of polychlorinated alkanes (Yan *et al.*, 2009). These represent the first strains isolated in pure culture to be able anaerobically to reductively dehalogenate 1,2,3-trichloropropane. For each of these strains, 1,2,3-trichloropropane was transformed to the intermediate allyl chloride (3-chloro-1-propene). Allyl chloride is unstable and underwent abiotic hydrolysis to form allyl alcohol. Allyl chloride also underwent abiotic reactions with sulfide and cysteine, reducing agents which were present in the growth medium, to form various other compounds, including diallyl sulfide, diallyl disulfide, allyl mercaptan and allyl methyl sulfide. These final products

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BL-DC-9^T and BL-DC-8 are EU679419 and EU679418, respectively.

Scanning electron and transmission electron micrographs of cells of strains BL-DC-9^T and BL-DC-8 and phenotypic characteristics of type species of genera in the phylum *Chloroflexi* are available with the online version of this paper.

are compounds that contribute to the recognizable odour associated with garlic (Laakso *et al.*, 1989; Lawson, 1996). The two strains were isolated from groundwater collected from a waste recovery well at the PetroProcessors of Louisiana, Inc. Superfund Site, near Baton Rouge, LA, USA, an area heavily contaminated with chlorinated solvents (Bowman *et al.*, 2006). Data from the present study show that strains BL-DC-9^T and BL-DC-8 represent a single novel species of a new genus in the phylum *Chloroflexi*.

The genomic DNA G+C content of strains BL-DC-8 and BL-DC-9^T was determined by HPLC as described by Mesbah *et al.* (1989) following DNA isolation by using an UltraClean microbial DNA isolation kit (MoBio Laboratories Inc.).

Cells for analysis of cellular fatty acids were grown in anaerobic basal medium prepared as described by Yan *et al.* (2009) except that the sodium sulfide solution was replaced with titanium citrate solution (Zehnder & Wuhrmann, 1976) as a reducing agent at a final concentration of 1.0 mM Ti(III) and 2.0 mM citrate. The medium was supplemented with 5 mM sodium acetate and 0.5 mM 1,1,2-trichloroethane, and provided with hydrogen gas (10%, v/v) in the headspace. Cells were harvested via centrifugation (10 000 g) following static incubation in the dark at 30 °C for 2 weeks. Cellular fatty acids were extracted, saponified and methylated according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed by using a gas chromatograph equipped with the Microbial Identification software package (Sasser, 1990).

Scanning electron microscopy was performed as described by Yan *et al.* (2009). For transmission electron microscopy, cells were collected and fixed in the same manner as for scanning electron microscopy. Preparations were rinsed with 0.02 M glycine in 0.1 M cacodylate buffer, post-fixed in 2% osmium tetroxide and stained with 0.5% uranyl acetate. Preparations were dehydrated with a graded ethanol series. The membrane was infiltrated with 1:1 ethanol/LR white embedding resin and infiltrated and embedded with 100% resin. Samples were sectioned with a DuPont MT5000 ultramicrotome and stained with Reynolds' lead citrate. Cross-sections were imaged with a JEOL 100CX transmission electron microscope.

Genomic DNA extraction, PCR amplification and 16S rRNA gene sequencing were performed as described by Yan *et al.* (2009). Phylogenetic analyses were performed by using the ARB program (Ludwig *et al.*, 2004). The neighbour-joining algorithm was used to build the phylogenetic tree, with Jukes–Cantor correction (Jukes & Cantor, 1969) followed by bootstrap analysis with PHYLIP 3.62 (Felsenstein, 1985) based on 1000 replications.

The genomic DNA G+C contents of strains BL-DC-8 and BL-DC-9^T were 54.0 and 53.8 mol%, respectively. The major cellular fatty acids included C_{18:1}ω9c, C_{16:1}ω9c,

Table 1. Cellular fatty acid profiles of strains BL-DC-8 and BL-DC-9^T

Values are percentages of the total fatty acids. –, Not detected.

Fatty acid	BL-DC-8	BL-DC-9 ^T
C _{9:0}	0.4	0.3
C _{10:0}	0.5	0.4
C _{12:0}	1.8	1.9
Anteiso-C _{14:0}	0.6	0.5
C _{14:0}	11.8	13.3
C _{15:0}	–	0.4
C _{16:1} ω9c	23.0	23.2
Summed feature 3*	2.7	2.1
C _{16:0}	17.1	20.8
Summed feature 5*	7.9	6.5
C _{18:1} ω9c	21.8	23.4
Summed feature 8*	4.7	–
C _{18:0}	7.1	7.1
C _{19:0} cyclo ω8c	0.7	–
C _{20:1} ω9c	–	0.6

* Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 contains one or more of C_{16:1}ω7c, C_{16:1}ω6c and iso-C_{15:0} 3-OH; summed feature 5 contains C_{18:2}ω6,9c and/or anteiso-C_{18:0}; summed feature 8 contains C_{18:1}ω7c and/or C_{18:1}ω6c.

C_{16:0} and C_{14:0} (Table 1). Apart from the presence of summed feature 8 (comprising C_{18:1}ω7c and/or C_{18:1}ω6c) as 4.7% of the total in strain BL-DC-8 and its absence from strain BL-DC-9^T, there were only minor differences in the cellular fatty acid contents of the two strains. Cells of the two strains were non-spore-forming, irregular cocci, 0.3–0.6 μm in diameter (see Supplementary Fig. S1 in IJSEM Online). Additional phenotypic characteristics were described previously (Yan *et al.*, 2009) and are included in the species description. The two strains shared nearly identical phenotypic properties, exhibiting identical substrate utilization profiles and differing only slightly in temperature range for growth (20–34 °C for strain BL-DC-8 and 20–37 °C for strain BL-DC-9^T). The two strains exhibited reductive dechlorination of 1,2-dichloropropane, a growth-related process, in the pH range 6.0–8.0 but not at pH ≤ 5.5 or ≥ 8.5 during a 2-month incubation period (Yan *et al.*, 2009). As the groundwater at the location from which the strains were isolated had a pH of 5.1 (Bowman *et al.*, 2006), the habitat of these strains probably extends to pH levels somewhat lower than determined in the laboratory test conditions employed by Yan *et al.* (2009).

Phylogenetically, strains BL-DC-8 and BL-DC-9^T were closely related, sharing 99.93% 16S rRNA gene sequence similarity (1 nt difference). In the neighbour-joining tree, the novel strains were placed distantly from recognized genera within a deep branch of the *Chloroflexi* (Fig. 1). Their closest cultured relatives were '*Dehalococcoides*'

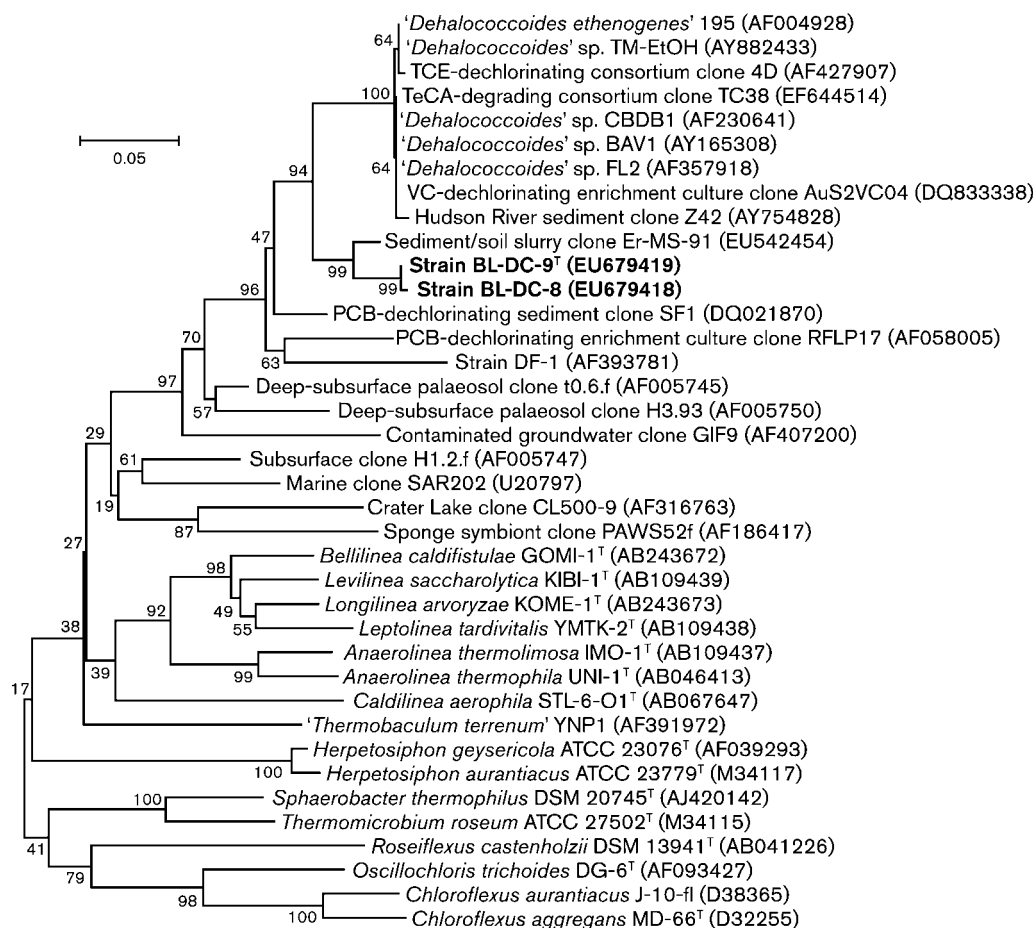


Fig. 1. Neighbour-joining dendrogram of the phylum *Chloroflexi* (Hugenholtz & Stackebrandt, 2004) based on 16S rRNA gene sequences showing the phylogenetic relationship of strains BL-DC-9^T and BL-DC-8 and related environmental clones and cultured isolates without validly published names to recognized taxa. Bootstrap values expressed as percentages of 1000 resamplings are shown at branch points. Bar, 5 substitutions per 100 nucleotide positions. PCB, Polychlorinated biphenyl; TCE, trichloroethene; TeCA, 1,1,2,2-trichloroethane; VC, vinyl chloride.

strains, but with only 90% 16S rRNA gene sequence similarity. The clustering of strains BL-DC-8 and BL-DC-9^T with the '*Dehalococcoides*' was supported by a bootstrap value of 94%.

Maymó-Gatell *et al.* (1997) informally proposed the name '*Dehalococcoides ethenogenes*' for a bacterial strain (strain 195) isolated from an enrichment culture derived from anaerobically digested wastewater sludge. This bacterium was the first strain isolated in pure culture that was shown to reductively dehalogenate tetrachloroethene to ethene. Additional strains with close phylogenetic relatedness to '*Dehalococcoides ethenogenes*' 195 (>99% 16S rRNA gene sequence similarity) have been reported subsequently in the literature as '*Dehalococcoides*' sp. (He *et al.*, 2003, 2005; Kube *et al.*, 2005; Sung *et al.*, 2006). There has been intense interest in this group of bacteria because of their ability to reductively dehalogenate a variety of chlorinated compounds that are widespread pollutants of environmental

importance (Hendrickson *et al.*, 2002; Bowman *et al.*, 2006). At the time of writing, however, the genus '*Dehalococcoides*' has not been formally described. The strain designated in the literature as '*Dehalococcoides ethenogenes*' 195 (Maymó-Gatell *et al.*, 1997) and the related '*Dehalococcoides*' strains BAV1, CBDB1, FL2 and GT (Adrian *et al.*, 2000; He *et al.*, 2003, 2005; Sung *et al.*, 2006) have not been deposited in public culture collections as required for valid publication under the Bacteriological Code (Lapage *et al.*, 1992), and no formal taxonomic descriptions for these exist. The above genus and species names, however, are widely used in the literature to the extent that '*Dehalococcoides*' has been carried to the class level with the informal proposal of Hugenholtz & Stackebrandt (2004) of the class '*Dehalococcoidetes*'. In order to move the taxonomy of this lineage forward and to establish an anchor point for future higher-level taxa, we propose the genus *Dehalogenimonas* gen. nov. to accommodate strains BL-DC-8 and BL-DC-9^T, this genus being

related most closely to but distinct from the 'Dehalococcoides' cluster.

Although strains BL-DC-8 and BL-DC-9^T share several common phenotypic features with the 'Dehalococcoides' strains reported to date, including strictly anaerobic respiration, requirement for H₂ as an electron donor, apparently exclusive use of halogenated compounds 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 (Adrian *et al.*, 2000; He *et al.*, 2003, 2005; Sung *et al.*, 2006; Yan *et al.*, 2009), they can be differentiated from members of the 'Dehalococcoides' cluster based on genotypic, chemotaxonomic and phenotypic properties. Based on 16S rRNA gene sequence comparisons, strains BL-DC-8 and BL-DC-9^T branch with but represent a distinct lineage at the 90 % similarity level from 'Dehalococcoides' strains (Fig. 1). Such a low level of 16S rRNA gene sequence similarity is indicative of a taxon distinct to at least the genus level. The genomic DNA G + C contents of strains BL-DC-8 and BL-DC-9^T (54.0 and 53.8 mol%, respectively) are appreciably higher than those for 'Dehalococcoides ethenogenes' 195 (48.9 mol%; Seshadri *et al.*, 2005), 'Dehalococcoides' sp. CBDB1 (47.0 mol%; Kube *et al.*, 2005) and 'Dehalococcoides' sp. BAV1 (47.1 mol%; http://genome.ornl.gov/microbial/deha_bav1/).

The cellular fatty acids determined for strains BL-DC-8 and BL-DC-9^T (Table 1) differ from those reported for 'Dehalococcoides' strains BAV1 and FL2, the only 'Dehalococcoides' strains characterized to date in this regard. Strains BL-DC-8 and BL-DC-9^T contained large proportions of C_{16:1}ω9c (23.0 and 23.2 %, respectively), significant concentrations of summed feature 5 (one or more of C_{18:2}ω6c, C_{18:2}ω9c and anteiso-C_{18:0}) (7.9 and 6.5 %, respectively) and summed feature 3 (one or more of C_{16:1}ω7c, C_{16:1}ω6c and iso-C_{15:0} 3-OH), while these were not detected in strain BAV1 or FL2 (White *et al.*, 2005). 10-Methyl C_{16:0} made up a large proportion of the cellular fatty acids in 'Dehalococcoides' sp. BAV1 and FL2 (21.3 and 24.4 %, respectively; White *et al.*, 2005) but was not detected in strain BL-DC-8 or BL-DC-9^T. C_{18:1}ω9c comprised a major fraction of the cellular fatty acids in strains BL-DC-8 and BL-DC-9^T (21.8 and 23.4 %, respectively) but was present in only trace amounts in 'Dehalococcoides' strains BAV1 and FL2 (0.4 and 0.5 %, respectively; White *et al.*, 2005).

Additionally, strains BL-DC-8 and BL-DC-9^T were shown to reductively dehalogenate polychlorinated alkanes (e.g. 1,2-dichloropropane, 1,2-dichloroethane, 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, 1,2,3-trichloropropane), but not monochlorinated alkanes (e.g. 1-chloropropane, 2-chloropropane), chlorinated alkenes (e.g. tetrachloroethene, trichloroethene, *cis*-dichloroethene, *trans*-dichloroethene, vinyl chloride) or chlorinated benzenes (1-chlorobenzene, 1,2-dichlorobenzene) (Yan *et al.*, 2009). All dechlorination reactions determined to date appear to involve exclusively a

dichlorelimination reaction mechanism (Yan *et al.*, 2009). This range of compounds serving as electron acceptors and the presence of apparently exclusive dichlorelimination (involving simultaneous removal of two chlorines from adjacent carbon atoms and formation of a carbon-carbon double bond) for strains BL-DC-8 and BL-DC-9^T differs from the 'Dehalococcoides' strains reported to date, which can reductively dehalogenate chlorinated alkenes and chlorinated benzenes and employ a reaction mechanism involving sequential removal of one chlorine atom at a time (Maymó-Gatell *et al.*, 1997, 1999, 2001; Adrian *et al.*, 2000; He *et al.*, 2003, 2005; Sung *et al.*, 2006).

Based on these characteristics, strains BL-DC-8 and BL-DC-9^T are considered to represent a new genus distinct from the informally proposed genus 'Dehalococcoides'. It should be noted that any future valid publication of the name 'Dehalococcoides' would not be impacted by, nor would it impact, the genus *Dehalogenimonas* proposed herein.

Levels of 16S rRNA gene sequence similarity between strains BL-DC-8 and BL-DC-9^T and members of recognized genera in the phylum *Chloroflexi* were extremely low, and phylogenetic distance readily served as a differentiating property (Fig. 1). Phenotypic characteristics also differentiated strains BL-DC-8 and BL-DC-9^T from recognized genera in the phylum *Chloroflexi* (Supplementary Table S1). Within the class *Anaerolinea*, members of the genera *Anaerolinea* (Sekiguchi *et al.*, 2003; Yamada *et al.*, 2006), *Bellilinea* (Yamada *et al.*, 2007), *Leptolinea* (Yamada *et al.*, 2006), *Levilinea* (Yamada *et al.*, 2006) and *Longilinea* (Yamada *et al.*, 2007) are filamentous, whereas cells of strains BL-DC-8 and BL-DC-9^T are irregular cocci. Additionally, members of the genera *Anaerolinea* and *Bellilinea* are thermophilic, while strains BL-DC-8 and BL-DC-9^T are mesophilic. At the time of writing, the class *Caldilineae* comprises a single genus with a single species, *Caldilinea aerophila* (Sekiguchi *et al.*, 2003; Yamada *et al.*, 2006), which can be differentiated from strains BL-DC-8 and BL-DC-9^T based on the fact that it is filamentous, thermophilic and facultatively aerobic (Supplementary Table S1).

Phenotypically, strains BL-DC-8 and BL-DC-9^T could be differentiated from the filamentous anoxygenic phototrophs of the genera *Chloroflexus*, *Chloronema*, *Heliolithrix*, *Oscillochloris* and *Roseiflexus* on the basis of cell morphology (Supplementary Table S1). In addition, the type strains of species of the genera *Chloroflexus*, *Heliolithrix* and *Roseiflexus* are thermophilic (Pierson & Castenholz, 1974; Pierson *et al.*, 1985; Hanada *et al.*, 1995, 2002) whereas strains BL-DC-8 and BL-DC-9^T are mesophilic (Yan *et al.*, 2009). Strains BL-DC-8 and BL-DC-9^T could be differentiated from the chemoheterotrophic genus *Herpetosiphon* on the basis that the latter has lower DNA G + C content and filamentous cell morphology and exhibits aerobic metabolism (Holt & Lewin, 1968; Lewin, 1970; Holt & Castenholz, 2001).

Strains BL-DC-8 and BL-DC-9^T may be differentiated from members of the class *Thermomicrobia* on the basis that the species assigned to this class, namely *Thermomicrobium roseum* (Perry, 2001; Hugenholtz & Stackebrandt, 2004) and *Sphaerobacter thermophilus* (Demharter *et al.*, 1989; Hugenholtz & Stackebrandt, 2004), are thermophilic, aerobic, rod-shaped and have appreciably higher DNA G+C contents. In addition, *Sphaerobacter thermophilus* is Gram-positive (Demharter *et al.*, 1989) whereas strains BL-DC-8 and BL-DC-9^T stain Gram-negative.

On the basis of phylogenetic, chemotaxonomic and phenotypic data, strains BL-DC-8 and BL-DC-9^T are clearly distinct from other genera in the phylum *Chloroflexi*. We suggest that strains BL-DC-8 and BL-DC-9^T represent a novel species of a new genus, for which the name *Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov. is proposed.

Description of *Dehalogenimonas* gen. nov.

Dehalogenimonas (De.ha.lo.ge.ni.mo'nas. L. prep. *de* away, off; N.L. n. *halogenum* halogen; L. fem. n. *monas* unit, monad; N.L. fem. n. *Dehalogenimonas* dehalogenating monad, reflecting the ability of these bacteria to dehalogenate chlorinated alkanes).

Cells are Gram-negative staining, non-motile, non-spore-forming, irregular cocci. Strictly anaerobic and mesophilic. Chemotrophic. Able to reductively dehalogenate chlorinated alkanes. Utilize H₂ as an electron donor. The G+C content of the genomic DNA is 53–54 mol%. On the basis of 16S rRNA gene sequence comparisons, the genus belongs to the phylum *Chloroflexi*. The type species is *Dehalogenimonas lykanthroporepellens*.

Description of *Dehalogenimonas lykanthroporepellens* sp. nov.

Dehalogenimonas lykanthroporepellens (ly.kan.thro.po.re.pel'lens. Gr. n. *lykanthropos* werewolf; L. part. adj. *repellens* repelling; N.L. part. adj. *lykanthroporepellens* repelling werewolves, because compounds exhibiting a pungent garlic aroma are produced when these organisms grow in the presence of 1,2,3-trichloropropane as an electron acceptor and sulfide as a reducing agent, garlic being said to repel werewolves in some fiction literature).

Cells are 0.3–0.6 µm in diameter. Able to reductively dehalogenate polychlorinated aliphatic alkanes, including 1,2,3-trichloropropane, 1,2-dichloropropane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane and 1,2-dichloroethane. When grown with 1,2,3-trichloropropane as an electron acceptor and sulfide as a reducing agent, the reactive intermediate allyl chloride is produced, which results in production of allyl alcohol, diallyl sulfide and diallyl disulfide. Does not utilize 1-chlorobenzene, 1-chloropropane, 2-chloropropane, 1,2-dichlorobenzene, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, tetrachloroethene, trichloroethene or vinyl chloride as electron

acceptors for growth. Growth is not supported by acetate, butyrate, citrate, ethanol, fructose, fumarate, glucose, lactate, lactose, malate, methanol, methyl ethyl ketone, propionate, pyruvate, succinate or yeast extract in the absence of H₂. Resistant to ampicillin and vancomycin. The temperature range for growth is 20–34 °C (optimum 28–34 °C). The pH range for growth is 6.0–8.0 (optimum pH 7.0–7.5). Growth occurs at NaCl concentrations up to at least 2% (w/v). Major cellular fatty acids are C_{18:1ω9c}, C_{16:1ω9c}, C_{16:0} and C_{14:0}. The G+C content of the genomic DNA of the type strain is 54.0 mol%.

The type strain, BL-DC-9^T (=ATCC BAA-1523^T =JCM 15061^T), was isolated from chlorinated solvent-contaminated groundwater at the PetroProcessors of Louisiana, Inc. Superfund Site, located near Baton Rouge, LA, USA. BL-DC-8, isolated from the same source, is a second strain of the species.

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