Dehalococcoides mccartyi gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and family *Dehalococcoidaceae* fam. nov., within the phylum *Chloroflexi*

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Six obligately anaerobic bacterial isolates (195^T, CBDB1, BAV1, VS, FL2 and GT) with strictly organohalide-respiring metabolisms were obtained from chlorinated solvent-contaminated aquifers, contaminated and uncontaminated river sediments or anoxic digester sludge. Cells were non-motile with a disc-shaped morphology, 0.3–1 μm in diameter and 0.1–0.2 μm thick, and characteristic indentations on opposite flat sides of the cell. Growth occurred in completely synthetic, reduced medium amended with a haloorganic electron acceptor (mostly chlorinated but also some brominated compounds), hydrogen as electron donor, acetate as carbon source, and vitamins. No other growth-supporting redox couples were identified. Aqueous hydrogen consumption threshold concentrations were <1 nM. Growth ceased when vitamin B_{12} was omitted from the medium. Addition of sterile cell-free supernatant of *Dehalococcoides*-containing enrichment cultures enhanced dechlorination and growth of strains 195 and FL2, suggesting the existence of so-far unidentified stimulants. Dechlorination occurred between pH 6.5 and 8.0 and over a temperature range of 15–35 °C, with an optimum growth temperature between 25 and 30 °C. The major phospholipid fatty acids were 14 : 0 (15.7 mol%), br15 : 0 (6.2 mol%), 16 : 0 (22.7 mol%), 10-methyl 16 : 0 (25.8 mol%) and 18 : 0 (16.6 mol%). Unusual furan fatty acids including 9-(5-pentyl-2-furyl)-nonanoate and 8-(5-hexyl-2-furyl)-octanoate were detected in strains FL2, BAV1 and GT, but not in strains 195^T and CBDB1. The 16S rRNA gene sequences

Abbreviations: DCE, dichloroethene; PCE, tetrachloroethene; PLFA, phospholipid fatty acid; TCE, trichloroethene.

Three supplementary tables are available with the online version of this paper.

of the six isolates shared more than 98 % identity, and phylogenetic analysis revealed an affiliation with the phylum *Chloroflexi* and more than 10 % sequence divergence from other described isolates. The genome sizes and G+C contents ranged from 1.34 to 1.47 Mbp and 47 to 48.9 mol% G+C, respectively. Based on 16S rRNA gene sequence comparisons, genome-wide average nucleotide identity and phenotypic characteristics, the organohalide-respiring isolates represent a new genus and species, for which the name *Dehalococcoides mccartyi* gen. nov., sp. nov. is proposed. Isolates BAV1 (=ATCC BAA-2100 =JCM 16839 =KCTC 5957), FL2 (=ATCC BAA-2098 =DSM 23585 =JCM 16840 =KCTC 5959), GT (=ATCC BAA-2099 =JCM 16841 =KCTC 5958), CBDB1, 195^T (=ATCC BAA-2266^T =KCTC 15142^T) and VS are considered strains of *Dehalococcoides mccartyi*, with strain 195^T as the type strain. The new class *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and family *Dehalococcoidaceae* fam. nov. are described to accommodate the new taxon.

Enrichment and isolation efforts with chlorinated ethenes and chlorinated benzenes as electron acceptors yielded unique, organohalide-respiring bacterial isolates from geographically distinct freshwater locations that shared more than 98 % 16S rRNA gene sequence similarity. The first isolate obtained dechlorinated tetrachloroethene (PCE) to vinyl chloride, ethene and inorganic chloride and was designated '*Dehalococcoides ethenogenes*' strain 195 (Maymó-Gatell *et al.*, 1997). Members of the '*Dehalococcoides*' group play key roles in the bioremediation of chlorinated solvent-contaminated sites, and consortia have been developed for bioaugmentation (Löffler & Edwards, 2006). Because of this successful industrial application, reductive dechlorination and organohalide respiration have been the subject of intense study for over a decade (Löffler *et al.*, 2013). The name '*Dehalococcoides*' has been used extensively in the literature and Hugenholtz & Stackebrandt (2004) have proposed the class '*Dehalococcoidetes*'; however, the genus '*Dehalococcoides*' has yet to be described. The intent of this work is the formal taxonomic description of the genus *Dehalococcoides*, and we present phenotypic, phylogenetic and genotypic characteristics of organohalide-respiring *Dehalococcoides* isolates including strains 195, BAV1, CBDB1, FL2, GT and VS. All strains were enriched from anoxic microcosms established with river sediments, aquifer materials or digester sludge (Table 1) amended with fermentable substrates (e.g. benzoate, pyruvate, lactate) or hydrogen as electron donor and a chlorinated ethene or a chlorinated benzene as electron acceptor. The enrichment process included repeated transfers (1–10 % inoculum, v/v) in reduced, completely synthetic mineral salts medium amended with acetate as the carbon source, hydrogen as electron donor (supplied in 2- to 200-fold excess of the theoretical electron equivalents required for reduction of the chlorinated electron acceptor), vitamins (Wolin *et al.*, 1964) and a chlorinated ethene (strains 195, BAV1, FL2, GT, VS) or a trichlorobenzene (strain CBDB1) as electron acceptor (Adrian *et al.*, 2000; He *et al.*, 2003, 2005; Maymó-Gatell *et al.*, 1997; Müller *et al.*, 2004; Sung *et al.*, 2006). Methanogenic archaea were eliminated by the addition of high concentrations of chlorinated solvents (up to 0.5 mM aqueous concentration) (strains BAV1, GT, 195) or 2–4 mM 2-bromoethanesulfonate (strains CBDB1, FL2 and VS). Selection against H_2/CO_2-metabolizing homoacetogens was accomplished by repeated transfers to medium amended with antibiotics that interfere with peptidoglycan biosynthesis [i.e. vancomycin (0.1 g l^{-1}) or ampicillin (0.05–3 g l^{-1})] or transfer to bicarbonate-free medium (strain VS) (Müller *et al.*, 2004) or medium with acetate as the only source of reducing equivalents (strain BAV1) (He *et al.*, 2002, 2003). The medium composition used for isolation and maintenance of the isolates varied slightly, and used different reductants including iron sulfide (FeS) (0.5 mM nominal concentration) (Ehrenreich & Widdel, 1994), a combination of sodium sulfide (0.2–0.5 mM), L-cysteine (0.2 mM) and DL-dithiothreitol (0.5–1 mM), or titanium(III) complexed with citrate or nitrilotriacetate (0.2–1.5 mM) (Moench & Zeikus, 1983; Zehnder & Wuhrmann, 1976). Isolation efforts used the dilution-to-extinction principle in liquid and semi-solid [0.5 % (w/v) low-melting-point agarose; gelling point <30 °C] medium (Adrian *et al.*, 2000; Löffler *et al.*, 2005). Dechlorinating activity was recovered from 10^{-5} to 10^{-7} dilution tubes and repeated dilution-to-extinction series yielded pure cultures of strains 195, VS, FL2 and GT. Tiny (<0.1 mm), opaque and barely visible colonies formed after 1.5 to 3 months in soft agar medium, and transfer of single colonies to fresh medium yielded pure cultures of strains BAV1 and CBDB1.

Culture purity was examined by multiple approaches including the generation of 16S rRNA gene clone libraries and sequencing of the inserts of multiple clones, quantitative enumeration of strain-specific reductive dehalogenase (RDase) genes (Ritalahti *et al.*, 2006; Sung *et al.*, 2006; Adrian *et al.*, 2007) and 16S rRNA genes (Ritalahti *et al.*, 2006), microscopic observation, growth experiments with alternate combinations of electron donor and acceptors and in rich media, as well as genome sequencing. The 16S rRNA genes of the isolates were highly similar, sharing more than 98 % sequence identity (Fig. 1). Phylogenetic analysis affiliated the sequences of the isolates with the phylum *Chloroflexi*, a deeply branching lineage within the domain *Bacteria*. This poorly characterized bacterial

Table 1. Utilization of chlorinated ethenes and other chloroorganic compounds by *Dehalococcoides* isolates

DCA, Dichloroethane; DCB, dichlorobenzene; DCE, dichloroethene; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzodioxin; PCE, tetrachloroethene; TCB, trichlorobenzene; TCE, trichloroethene; TeCB, tetrachlorobenzene; VC, vinyl chloride. ND, Not determined. Also indicated are genes that encode RDases with assigned catalytic function detected in the individual strains.

Strain	Origin	Electron acceptor(s) used for isolation	Chlorinated ethenes used as electron acceptors	Chlorinated ethenes co-metabolized	Major end product(s)	Substrate range*	RDase gene(s) with assigned function (catalytic activity)
195^T	Digester sludge, Ithaca, NY, USA	PCE	PCE, TCE, *cis*-DCE, 1,1-DCE	*trans*-DCE, VC	VC, ethene	1,2-DCA, 1,2-dibromoethane, PCBs, PCDDs, chlorinated naphthalenes, chlorobenzenes chlorophenols	*pceA* (PCE→TCE), *tceA* (TCE→VC)
CBDB1	Saale River sediment, Germany	1,2,3-TCB and 1,2,4-TCB	PCE, TCE	None	*trans*-DCE (*cis*-DCE)	Chlorobenzenes, chlorophenols, PCDDs, PCBs	*cbrA* (1,2,3,4-TeCB→1,2,4-TCB, 1,2,3-TCB→1,3-DCB)
BAV1	Contaminated aquifer, Oscoda, MI, USA	*cis*-DCE, VC	*cis*-DCE, *trans*-DCE, 1,1-DCE, VC	PCE, TCE	Ethene	1,2-DCA, vinyl bromide	*bvcA* (DCEs, VC→ethene)
VS	Contaminated aquifer, Victoria, TX, USA	1,1-DCE, VC	TCE, *cis*-DCE, 1,1-DCE, VC	None	Ethene	ND	*vcrA* (DCEs, VC→ethene)
GT	Contaminated aquifer, Cottage Grove, WI, USA	TCE	TCE, *cis*-DCE, 1,1-DCE, VC	None	Ethene	ND	*vcrA* (DCEs, VC→ethene)
FL2	Red Cedar River, Okemos, MI, USA	TCE	TCE, *cis*-DCE, *trans*-DCE	PCE, VC	VC, ethene	ND	*tceA* (TCE→VC)

*Dehalogenation was observed, but growth with these compounds has not been demonstrated unequivocally. The range of halogenated compounds dehalogenated by individual strains has not been evaluated systematically.

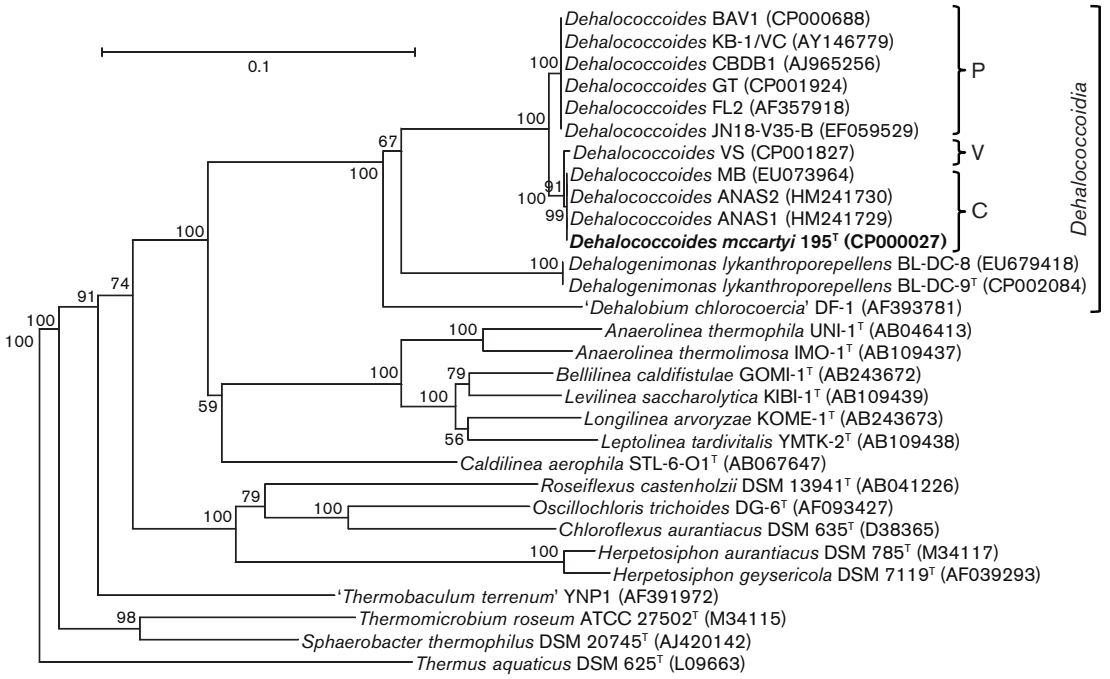

Fig. 1. Phylogeny of *Dehalococcoides mccartyi* gen. nov., sp. nov. 195^T (in bold) and related *Dehalococcoides* isolates among the isolated members of *Chloroflexi* based on nearly complete 16S rRNA gene sequences. The three recognized *Dehalococcoides* subgroups, Pinellas (P), Cornell (C) and Victoria (V), are indicated with braces. The tree was calculated using maximum-likelihood estimation. *Chloroflexus aurantiacus* DSM 635^T is the type strain of the type species of the genus *Chloroflexus*; *Thermus aquaticus* DSM 625^T served as the outgroup. An unrooted tree of the *Chloroflexi* was prepared using CLUSTAL W and an IUB cost matrix (gap open cost=10, gap extend cost=6.66), with bootstrap values greater than 50 % expressed as percentages of 1000 replications. Bar, 10 substitutions per 100 base pairs.

phylum encompasses a phylogenetically diverse collection of 16S rRNA gene sequences and is separated into at least six major lineages including the *Anaerolineae*, *Caldilineae*, *Dehalococcoidia* (proposed in this study), the marine SAR202 cluster (without cultured representatives), 'traditional' *Chloroflexi* and the *Thermomicrobia* (Hugenholtz & Stackebrandt, 2004; Yamada *et al.*, 2006). The class '*Dehalococcoidetes*' was informally introduced to accommodate organohalide-respiring bacteria related to '*Dehalococcoides ethenogenes*' strain 195 (Hugenholtz & Stackebrandt, 2004). The *Dehalococcoides* isolate 16S rRNA gene sequences, together with a large number of environmental and mixed-culture clone sequences, form a tight cluster, which Hendrickson *et al.* (2002) divided into three phylogenetic subgroups based on sequence signatures in the hypervariable V2 and V6 regions of the 16S rRNA gene. The 'Cornell' subgroup includes strains 195 and MB (Cheng & He, 2009), the 'Victoria' subgroup includes strain VS and the 'Pinellas' subgroup comprises the other available *Dehalococcoides* isolates, as well as most cultured *Dehalococcoides* strains identified in mixed cultures and the majority of the environmental clone sequences (Fig. 1). Members of the 'Pinellas' subgroup share identical or highly similar (1–3 nucleotide differences) 16S rRNA gene sequences, and 14–16 and 23–24 base differences distinguish the 16S rRNA genes of members of the Pinellas subgroup from members of the Victoria and Cornell subgroups, respectively. In addition to *Dehalococcoides*, the class *Dehalococcoidia* encompasses two other organohalide-respiring clusters, represented by the isolate '*Dehalobium chlorocoercia*' DF-1 and *Dehalogenimonas lykanthroporepellens* BL-DC-8 and BL-DC-9^T (Fig. 1).

The *Dehalococcoides* isolates exhibited a highly restricted catabolic range, and only certain chlorinated and brominated compounds served as respiratory electron acceptors (Table 1). All isolates required hydrogen as the electron donor, which could not be replaced by formate. Acetate served as a carbon source. Fermentative growth was not observed, and no other growth-supporting electron donor/acceptor combinations were identified. Comparative genome analysis of strains 195, CBDB1, BAV1, GT and VS (Kube *et al.*, 2005; Seshadri *et al.*, 2005; McMurdie *et al.*, 2009) identified no other respiratory or fermentative energy-conserving pathways, thus corroborating the results from physiological studies that members of this bacterial group are strictly organohalide-respiring bacteria. Cytochromes were absent. Dechlorination and growth occurred between pH 6 and 8, with highest activity measured between pH 6.9 and 7.5. All isolates were mesophilic, and dechlorination was

measured at incubation temperatures ranging from 15 to 35 °C, with optimum growth occurring at 25–30 °C, except for strain 195, which was cultured from an anoxic digester and had an optimum near 35 °C. No dechlorination occurred at 4 or 40 °C, but cultures stored at 4 °C or at room temperature for several months recovered dechlorination activity after prolonged lag times. Spores were not found, and incubation at 45 °C or above resulted in loss of dechlorination activity and viability. The lag time before dechlorination started following 1–5 % (v/v) transfers under optimum temperature and pH conditions ranged from a few days to 2–6 weeks, depending on the reductant and the age of the inoculum. The shortest lag times were observed in medium reduced with FeS and/or titanium (III). Similar dechlorination performance and growth were observed when incubated under static conditions or with modest agitation. The doubling times and growth yields of the isolates ranged from 0.8 to 3 days and 6.3×10^7 to 3.1×10^8 cells per μmol chloride released, respectively (Table 2). Although densities of 10^7–10^8 cells per ml culture fluid were achieved, turbidity was too low to be monitored via optical density measurements. The low biomass yield of about 1 mg (wet wt) from 100 ml culture suspension has hampered detailed biochemical studies and prevented certain chemotaxonomic analyses (e.g. cell-wall composition) typically included in the description of a novel genus.

Microscopic analysis of cell suspensions revealed that the cells were small and shared unusual morphology features. Suspended cells were visible by phase-contrast microscopy for brief moments but then 'disappeared' from the field of view, apparently due to a disc-shaped morphology with a thickness of less than 0.2 μm, which is the limit of the light microscope's resolving power. Transmission and scanning electron microscopy confirmed round, disc-shaped cells, no more than 1 μm wide and 0.1–0.2 μm thick, and characteristic biconcave indentations on opposite flat sides of the cell were visible (Fig. 2a–d). The estimated volume of a single *Dehalococcoides* cell is 0.02 μm^3, which is roughly 30-fold lower than the mean *Escherichia coli* cell volume of 0.6–0.7 μm^3 (Duhamel *et al.*, 2004; Kubitschek, 1990; Löffler *et al.*, 2013), or about twice the cell volume of '*Pelagibacter ubique*' (SAR11) (Rappé *et al.* 2002). Scanning electron microscopy revealed unusual cell surface features

Table 2. Experimental growth yields and doubling times of various *Dehalococcoides* strains and related dechlorinating *Chloroflexi* isolates

Strain	Electron acceptor*	Doubling time (days)	Yield per μmol Cl^- released		Reference
			Cells	Dry weight (g)†	
Dehalococcoides mccartyi gen. nov., sp. nov.					
Strain 195^T	PCE	0.8	2.9×10^8	3.5	Maymó-Gatell *et al.* (1997)
	2,3-DCPh	ND	8.3×10^7	1.0	Adrian *et al.* (2007)
Strain CBDB1	HCB	ND	8.2×10^7	1.1	Jayachandran *et al.* (2003)
	2,3-DCPh	2–3	7.6×10^7	0.91	Adrian *et al.* (2007)
	PCE	2–3	1.3×10^8	1.6	Marco-Urrea *et al.* (2011)
Strain BAV1	VC	2.2	6.3×10^7	0.76	He *et al.* (2003)
Strain FL2	TCE	2.4	7.8×10^7	0.95	He *et al.* (2005)
	cis-DCE	ND	8.4×10^7	1.0	He *et al.* (2005)
	trans-DCE	ND	8.1×10^7	0.98	He *et al.* (2005)
Strain GT	VC	2–2.5	2.5×10^8	3.0	Sung *et al.* (2006)
	TCE	2–2.5	3.1×10^8	3.8	Sung *et al.* (2006)
Strain VS	VC	1.7‡	5.2×10^8‡	6.2	Cupples *et al.* (2003)
Strain MB	TCE	1.0	8.6×10^7	1.1	Cheng & He (2009)
Dehalogenimonas lykanthroporepellens BL-DC-9^T	1,2,3-TCP	4.1	2.9×10^6	ND	Yan *et al.* (2009)
	1,2-DCP	ND	1.5×10^7	ND	Yan *et al.* (2009)
'*Dehalobium chlorocoercia*' DF-1	2,3,4,5-PCB	2.0	1.1×10^8	ND	May *et al.* (2008)

*DCE, Dichloroethene; DCP, dichloropropane; DCPh, dichlorophenol; HCB, hexachlorobenzene; PCB, polychlorinated biphenyl; PCE, tetrachloroethene; TCE, trichloroethene; TCP, trichloropropane; VC, vinyl chloride.

†16S rRNA gene-targeted qPCR competitive PCR (strain VS) or microscopic cell count (strain CBDB1) data were converted to g dry weight using a conversion factor of 1.2×10^{-14} g per cell. This conversion factor was calculated assuming that cells are cylinders (0.5 μm diameter and 0.2 μm thick), contain 70 % water and have a single 16S rRNA gene copy per genome (i.e. per cell).

‡Determined in a highly enriched mixed culture.

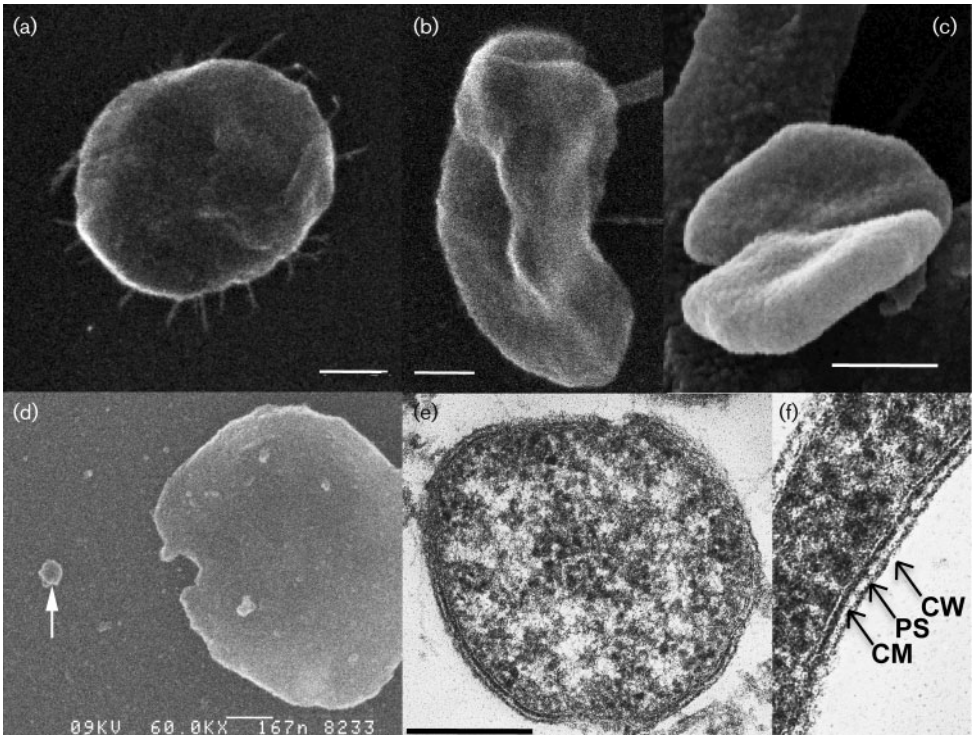

Fig. 2. Electron micrographs showing characteristic features of *Dehalococcoides mccartyi* strains. (a–d) Scanning electron micrographs depict cellular appendages of strain BAV1 (a), demonstrate the disc morphology with characteristic biconcave indentations on opposite flat sides of the cell (strains BAV1 and CBDB1) (b and c, respectively) and visualize characteristic notches (strain GT) (d), which were commonly observed in *Dehalococcoides* pure cultures. Small, round blebs about 50–100 nm in diameter occurred, often in proximity to the notches (arrow in d). (e, f) Transmission electron micrograph of a cross-section of strain 195^T, showing the cell-wall (CW) architecture [magnification in (f) is twice that in (e)]. Arrows indicate the S-layer-like CW, the periplasmic space (PS) and the cell membrane (CM). The images of strains BAV1 and GT (a, b, d) are reprinted with permission from He *et al.* (2003) and Sung *et al.* (2006), and those of strain 195^T (e, f) were adapted, with permission, from Maymó-Gatell *et al.* (1997). The micrograph of strain CBDB1 (c) was taken by Jörg Wecke, Robert Koch-Institut Berlin, Germany, and L. A. Bars, 0.2 μm (a, c, e), 0.111 μm (b) and 0.167 μm (d).

such as filamentous appendages (Fig. 2a). The function of these appendages is unclear, but they may play a role in attachment of cells to each other or to surfaces, which has been observed in laboratory and field studies (Lendvay *et al.*, 2003; Amos *et al.*, 2009). Motility was never observed, and no growth occurred on agar surfaces. The addition of vancomycin and ampicillin did not prevent dechlorination or growth through several subsequent transfers, suggesting that the *Dehalococcoides* isolates were resistant to antibiotics that interfere with peptidoglycan biosynthesis. This finding suggested an unusual cell-wall structure, a hypothesis supported by the observations that the cell wall of strain 195 did not react with peptidoglycan-specific lectin (Maymó-Gatell *et al.*, 1997). Transmission electron micrographs revealed an unusual cell-wall ultrastructure (Fig. 2e, f) that resembled the S-layer cell walls of archaea (Kandler, 1993), with no discernible peptidoglycan layer as typically seen in micrographs of bacteria. A 105–110 kDa protein (DET1407; GenBank accession number AAW39334) has been proposed to be an S-layer component (Morris *et al.*, 2006).

The genome sequences of *Dehalococcoides* strains 195, BAV1, CBDB1, GT and VS are available (Table S1, available in IJSEM Online), and genome analyses corroborated the morphological and physiological observations. For example, genes encoding hydrogenase and reductive dehalogenase protein complexes were present, but genes implicated in sulfate, nitrate or fumarate reduction were absent. Genes encoding cytochrome-containing proteins and cytochrome biosynthesis were absent. Therefore, the genome analysis supported experimental evidence that *Dehalococcoides* strains are restricted to cytochrome-independent organohalide respiration as the only means of energy conservation. Motility genes, sporulation genes and genes for peptidoglycan biosynthesis were not identified.

Oxygen exposure irreversibly inhibited dechlorination, growth and viability (Amos *et al.*, 2008), and strictly anoxic techniques for culture manipulations were vital. For example, plastic syringes were stored inside anoxic chambers for several days or filled with sterile solutions

of reductants [i.e. 1 mM Na_2S or 1 mM titanium (III)] prior to flushing with oxygen-free N_2 and use. The redox potential of the medium also affected dechlorination and viability, and successful cultivation required the addition of reductants. Cultures with redox potentials above −110 mV (i.e. redox indicator resazurin pink), but without measurable oxygen, did not exhibit dechlorination activity, even after the addition of reductant(s). Interestingly, growth of strain CBDB1 in the titanium (III)-reduced medium devoid of any amended sulfur compound was sustained, suggesting that impurities of reagent-grade chemicals provide sufficient sulfur to fulfil the growth requirements of *Dehalococcoides* strains.

To achieve standardized growth conditions for the available *Dehalococcoides* isolates, a MOPS-buffered medium containing the following components (per litre) was devised: 1 g NaCl (17 mM), 0.41 g $MgCl_2 . 6H_2O$ (2 mM), 0.27 g NH_4Cl (5 mM), 0.52 g KCl (7 mM), 0.15 g $CaCl_2 . 2H_2O$ (1 mM), 0.2 g KH_2PO_4 (1.5 mM), 0.25–1 mg resazurin (1–4.4 μM), 0.41 g sodium acetate (5 mM), 1 ml trace element solution (1000× stock) and 1 ml selenite-tungstate solution (1000× stock) (Löffler *et al.*, 1999). The medium was boiled and cooled to room temperature under a stream of N_2 before the following components were added (final concentrations per litre): 0.084 g $NaHCO_3$ (1 mM), 0.048 g $Na_2S . 9H_2O$ (0.2 mM) and 0.031 g L-cysteine (0.2 mM). The medium was dispensed into N_2-flushed 20- to 160-ml glass vials or serum bottles and closed with thick butyl-rubber stoppers (Geo-Microbial Technologies or Bellco Glass) or Teflon-lined butyl-rubber septa (Ochs) and aluminium crimps. After autoclaving at 121 °C for 30 min, the following additions were made: 20 mM filter-sterilized sodium MOPS (1 M stock, adjusted to pH 7.4 with 1 M HCl), 0.5 mM titanium (III) nitrilotriacetate (25 mM stock, filter-sterilized), vitamins [0.5 ml three-vitamin 2000× stock, 1.0 ml six-vitamin 1000× stock and 1.0 ml cyanocobalamin 1000× stock; all filter-sterilized (Rosner *et al.*, 1997)]. Sterile hydrogen gas (2–20 % of headspace, v/v) was added using a syringe filter (0.2 μm). The final medium had a pH of 7.1–7.2 and was reduced (i.e. resazurin colourless) and without precipitate. All *Dehalococcoides* strains grew in this medium, and cultures were transferred (2 % inoculum, v/v) to fresh medium every 2–10 weeks.

To generate biomass for lipid analysis, the *Dehalococcoides* isolates were grown in this MOPS-buffered medium and amended with two or three feedings of substrate [i.e. PCE for strain 195; trichloroethene (TCE) for strain GT and FL2; *cis*-dichloroethene (DCE) for strain BAV1; an equimolar 1,2,3-/1,2,4-trichlorobenzene mixture for strain CBDB1] to achieve dehalogenation of a total amount of about 100 μmol chlorinated electron acceptor. When about 80–90 % of the final electron acceptor amendment was consumed, the bottles were shipped via overnight carrier to Microbial Insights (http://www.microbe.com/). The total lipid dissolved in chloroform was fractionated, and the phospholipid fraction was analysed for phospholipid fatty acid (PLFA) profiles (White *et al.*, 2005). The cellular fatty acids of the *Dehalococcoides* strains analysed included large proportions of even-carbon-number, straight-chain and mid-chain-branched, saturated fatty acids, which comprised 51.8–64.3 % and 17.4–43.5 %, respectively, of the total PLFA (Table S3). These values are similar to those reported previously for strain BAV1 (68.8 %) and strain FL2 (59.5 %) grown in a different mineral salts medium (White *et al.*, 2005). The major PLFA were 14 : 0 (15.7 mol%), br15 : 0 (6.2 mol%), 16 : 0 (22.7 mol%), 10-methyl 16 : 0 (25.8 mol%) and 18 : 0 (16.6 mol%). Non-branched monounsaturated PLFA were present in low abundances in strains FL2, BAV1 and GT (<2.5 mol%), and larger amounts were detected in strain CBDB1 (<7.2 mol%) and strain 195 (10.4 mol%). In a previous analysis, no monounsaturated PLFA were detected in strains FL2 and BAV1; however, unusual furan fatty acids including 9-(5-pentyl-2-furyl)-nonanoate (Fu18 : 2ω6) and 8-(5-hexyl-2-furyl)-octanoate (Fu18 : 2ω6) were found (White *et al.*, 2005). The current study corroborated the presence of two 18-carbon furan PLFA in strains FL2 (3.7 mol%), BAV1 (2.0 mol%) and GT (7.7 mol%), although these unusual furan fatty acids were not detected in strain CBDB1 or strain 195. Odd-carbon-number furan fatty acids were not detected.

Phenotypic properties including a non-phototrophic, strictly anaerobic and mesophilic metabolism restricted to organohalide respiration, G+C content and a unique cell morphology distinguish members of the *Dehalococcoidia* from cultured representatives of all other lineages currently affiliated with the phylum *Chloroflexi* (discussed by Moe *et al.*, 2009). The *Dehalococcoidia* currently comprises three lineages: *Dehalococcoides*, represented by the six isolates described herein, strain MB (Cheng & He, 2009) and strains ANAS1 and ANAS2 recently obtained from a trichloroethene-to-ethene-dechlorinating consortium (Lee *et al.*, 2011), the genus *Dehalogenimonas* (represented by *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9^T), and a separate branch represented by a single isolate informally named '*Dehalobium chlorocoercia*' strain DF-1 (Fig. 1). The cultured *Dehalococcoidia* depend strictly on organohalide respiration to conserve energy for maintenance and growth; however, key differences exist between the three currently known branches. The *Dehalococcoides* share ~90 and ~87.5 % 16S rRNA gene sequence identity, respectively, with *Dehalogenimonas lykanthroporepellens* and '*Dehalobium chlorocoercia*'. The *Dehalococcoides* strains share properties with the two described *Dehalogenimonas* strains, but genotypic, chemotaxonomic and phenotypic features distinguish the two groups. Aside from 10–11 % 16S rRNA gene sequence divergence, the G+C content of the *Dehalococcoides* genomes is 6–8 % lower than the 55 mol% G+C determined for the *Dehalogenimonas lykanthroporepellens* genome and 10–12 % lower than the G+C content of the '*Dehalobium chlorocoercia*' genome (Table S1). While *Dehalogenimonas lykanthroporepellens* BL-DC-8 and BL-DC-9^T were reported to reduce electron acceptors via a dihaloelimination mechanism (i.e. two

chlorine atoms are eliminated from adjacent, saturated carbon atoms to yield an alkene in a two-reducing-equivalent reduction), the six *Dehalococcoides* strains carry out hydrogenolysis and, in the case of some substrates like 1,2-dichloroethane, dihaloelimination (except strain CBDB1). The *Dehalococcoides* strains were irreversibly inhibited by brief oxygen exposure, whereas strains BL-DC-8 and BL-DC-9^T were more oxygen tolerant (Yan *et al.*, 2009). '*Dehalobium chlorocoercia*' is represented by a single strain, designated DF-1 (May *et al.*, 2008), and the genus and species names have yet to be validly published. Distinguishing features of strain DF-1 from the *Dehalococcoides* strains include 10 % 16S rRNA gene sequence divergence, a smaller cell size of 137 ± 51 nm thickness and 75–339 nm diameter, sheaths or capsules surrounding the cells, aggregate growth, the use of formate as an electron donor, and susceptibility to antibiotics that interfere with peptidoglycan biosynthesis. Further, strain DF-1 cannot be grown in defined medium and requires co-cultivation with a *Desulfovibrio* strain or the addition of a *Desulfovibrio* strain cell extract (May *et al.*, 2008).

Results from whole-genome comparisons (Table S2) are consistent with the proposal to include all described *Dehalococcoides* strains in a single species of the new genus *Dehalococcoides* gen. nov. Strains BAV1, CBDB1 and GT show high genome-aggregate average nucleotide identities (ANI) (>98.7 %). These values are well above the 95 % ANI that corresponds to the 70 % DNA–DNA hybridization standard that is frequently used for species demarcation (Goris *et al.*, 2007), revealing that these strains show a high degree of genomic relatedness. Strains BAV1, CBDB1 and GT show lower ANI values, 86–87 %, to strains VS and 195 (Table S2). However, the gene-content differences among the most phylogenetically divergent pairs of genomes are comparable to, if not smaller than (e.g. BAV1/VS vs BAV1/GT comparison), the gene-content differences seen among the most closely related genomes. The application of a *Dehalococcoides* pangenome microarray confirmed extensive gene-content similarities experimentally between the different strains (Hug *et al.*, 2011). The five sequenced *Dehalococcoides* genomes carry a similar number of strain-specific genes, regardless of the degree of their evolutionary divergence, and these genes are restricted to the RDase and accessory genes, as well as hypothetical genes, encoded in genomic islands and mobile genetic elements (Kube *et al.*, 2005; Seshadri *et al.*, 2005; McMurdie *et al.*, 2009). Although the genome sequences of strains FL2, MB, ANAS1 and ANAS2 are not available, microarray studies suggest that they contain the same characteristic *Dehalococcoides* core genes (Hug *et al.*, 2011; Lee *et al.*, 2011). The presence/absence of RDase operons reflect the main difference in gene content between the *Dehalococcoides* strains. The high gene-content similarity indicates that these *Dehalococcoides* strains share overlapping ecological niches and are under similar ecological constraints; otherwise, the strain-specific environmental settings would have selected for different gene contents. This contrasts with observations for members of other genera such as *Escherichia*, *Shewanella* and *Burkholderia*, which show similar evolutionary relatedness (e.g. share similar ANI values) to each other to that shown by the *Dehalococcoides* strains, but are more versatile and diverse, and therefore share fewer functional genes (Konstantinidis *et al.*, 2006). Based on their high shared gene content and genomic relatedness, as well as unifying morphological and physiological characteristics, we propose that the *Dehalococcoides* strains should be included within the same species.

The sensitivity to oxygen and redox potential limits the distribution of *Dehalococcoides* to environments where strictly anoxic conditions prevail. Not surprisingly, all *Dehalococcoides* isolates were derived from anoxic environments, including digester sludge, sediments and aquifers. Except for strain FL2, the *Dehalococcoides* isolates were obtained from contaminated environments. The quest for microbes capable of chlorinated compound transformation has introduced a strong bias towards sampling polluted sites, but the isolation of strain FL2 from a river sediment with no reported contamination with halogenated compounds demonstrated that *Dehalococcoides* strains are members of natural microbial communities not impacted by anthropogenic contamination. The obvious question that arises is, how do strictly organohalide-respiring *Dehalococcoides* survive in pristine environments and conserve energy for cell maintenance and growth? A plausible explanation is the use of naturally occurring organohalides, which have been observed in a variety of habitats including marine ecosystems, sediments, subsurface environments and soils (Öberg, 2002; Gribble, 2003; Krzmarzick *et al.*, 2012). The production of organohalogens in soils has been demonstrated (de Jong *et al.*, 1994; Hoekstra *et al.*, 1999), and anoxic microenvironments occur in unsaturated soils (Schink, 2006), suggesting that the habitat range of *Dehalococcoides* probably extends beyond anoxic sediments, sludges and saturated aquifers, which have been the source materials for enrichment efforts to date. Moreover, the presence of 12–36 sets of genes predicted to encode RDases indicates that this genus is highly adapted to use naturally occurring as well as anthropogenic organohalogens.

Phylogenetic, phenotypic and genomic characteristics distinguish the *Dehalococcoides* isolates from other genera in the phylum *Chloroflexi*, including other organohalide-respiring populations (i.e. *Dehalogenimonas lykanthroporepellens* and '*Dehalobium chlorocoercia*'; May *et al.*, 2008; Moe *et al.*, 2009). We propose the new genus, *Dehalococcoides* gen. nov., with *Dehalococcoides mccartyi* sp. nov. as the type species, to accommodate the new isolates. The type species of the genus, *Dehalococcoides mccartyi*, was formerly designated '*Dehalococcoides ethenogenes*'. *Dehalococcoides mccartyi* is embedded in the family *Dehalococcoidaceae* fam. nov., of the order *Dehalococcoidales* ord. nov., of the class *Dehalococcoidia* classis nov. Based on phylogenetic, physiological, morphological and genomic differences, we propose that *Dehalogenimonas* and '*Dehalobium*' represent distinct genera within the *Dehalococcoidia* (Kittelmann & Friedrich, 2008; Hugenholtz & Stackebrandt, 2004).

Description of *Dehalococcoides* gen. nov.

Dehalococcoides [De.ha.lo.coc.co.i′des. L. prep. *de* away, off; N.L. pref. *halo-* (from N.L. n. *halogenum*) halogen; N.L. masc. n. *coccus* (from Gr. masc. n. *kokkus* grain, seed) coccus; L. suff. *-oides* resembling, similar; N.L. masc.n. *Dehalococcoides* coccus-shaped dehalogenating organism].

Strictly hydrogenotrophic, organohalide-respiring metabolism. Strict anaerobes. Cells are non-motile, non-spore-forming, non-pigmented and disc-shaped. Possess multiple reductive dehalogenase genes. Cytochromes are absent. Hydrogen is consumed to threshold concentrations below 1 nM. Resistant to antibiotics that interfere with peptidoglycan biosynthesis. Cells stain Gram-indifferent and peptidoglycan in the cell wall is absent. Even-carbon-number, straight-chain saturated fatty acids (14 : 0, 16 : 0 and 18 : 0) and the methyl-branched fatty acid 10-methyl 16 : 0 dominate the PLFA profile. 16S rRNA gene sequence comparisons affiliate the genus with the phylum *Chloroflexi*. The type species is *Dehalococcoides mccartyi*.

Description of *Dehalococcoides mccartyi* sp. nov.

Dehalococcoides mccartyi (mc.car′ty.i. N.L. gen. masc. n. *mccartyi* of McCarty, in honour of Dr Perry L. McCarty for his visionary contributions to environmental science and engineering, engineering practice and education, including the field of microbial reductive dehalogenation).

Displays the following characteristics in addition to those described for the genus. Catalyses the reductive dehalogenation of mono- and polychlorinated and brominated aromatic compounds, alkanes and alkenes. Specific reductive dehalogenase genes distinguish strains and confer distinct dechlorination capabilities. The G+C content of genomic DNA ranges from 47 to 48.9 mol%. Disc-shaped cells are 0.3–1 μm wide and 0.1–0.2 μm thick. No colony formation occurs on agar surfaces. Chemotrophic. Acetate serves as a carbon source. Growth requires reducing conditions and vitamin B_{12}. Oxygen and sulfite cause irreversible inhibition. Genome size ranges from 1.34 to 1.47 Mb.

The type strain, 195^T (=ATCC BAA-2266^T =KCTC 15142^T), was isolated from an anaerobic reactor seeded with digester sludge from a wastewater treatment plant at Ithaca, NY, USA. Previously described as '*Dehalococcoides ethenogenes*' strain 195.

Description of *Dehalococcoidaceae* fam. nov.

Dehalococcoidaceae (De.ha.lo.coc.coi.da′ce.ae. N.L. fem. pl. n. *Dehalococcoides* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Dehalococcoidaceae* family of the genus *Dehalococcoides*).

The description is the same as for the genus *Dehalococcoides*. The type genus is *Dehalococcoides* gen. nov.

Description of *Dehalococcoidales* ord. nov.

Dehalococcoidales (De.ha.lo.coc.coi.da′les. N.L. fem. pl. n. *Dehalococcoides* type genus of the order; *-ales* ending to denote an order; N.L. fem. pl. n. *Dehalococcoidales* order of the genus *Dehalococcoides*).

Cells are non-motile, non-spore-forming, strictly anaerobic and mesophilic. Members of the order are only known to utilize halogenated organics as electron acceptors and H_2 as the electron donor. The type genus is *Dehalococcoides* gen. nov. Also contains the genera *Dehalogenimonas* and '*Dehalobium*'.

Description of *Dehalococcoidia* classis nov.

Dehalococcoidia (De.ha.lo.coc.co.i′di.a. N.L. *Dehalococcoides* type genus of the type order of the class; suff. *-ia* ending to denote a class; N.L. neut. pl. n. *Dehalococcoidia* the *Dehalococcoides* class).

The class *Dehalococcoidia* is defined based on phylogenetic analysis and comparison of the 16S rRNA gene sequences of 11 strains, including the six isolates described herein, belonging to the genus *Dehalococcoides*, two isolates of the genus *Dehalogenimonas*, one isolate of '*Dehalobium*' and numerous uncultured representatives. The type order is *Dehalococcoidales* ord. nov.

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