Brooklawnia cerclae gen. nov., sp. nov., a propionate-forming bacterium isolated from chlorosolvent-contaminated groundwater

Hee-Sung Bae,¹ William M. Moe,² Jun Yan,² Igor Tiago,³ Milton S. da Costa⁴ and Fred A. Rainey¹

Correspondence
William M. Moe
moemwil@lsu.edu

^{1,2}Department of Biological Sciences¹ and Department of Civil and Environmental Engineering², Louisiana State University, Baton Rouge, LA 70803, USA

³Departamento de Zoologia and Centro de Neurociências, Universidade de Coimbra, 3004-517 Coimbra, Portugal

⁴Departamento de Bioquímica and Centro de Neurociências, Universidade de Coimbra, 3001-401 Coimbra, Portugal

Two novel facultatively anaerobic bacterial strains, BL-34^T and BL-35, isolated from groundwater contaminated by a mixture of chlorosolvents were characterized using a polyphasic approach. The two strains exhibited essentially identical taxonomic features except for a vitamin B₁₂ requirement by strain BL-35 for optimal growth. Phylogenetically, the isolates were affiliated with members of the family *Propionibacteriaceae* and were placed in a phylogenetic branch adjacent to, but distinct from, those of the genera *Propionimicrobium*, *Propionibacterium*, *Luteococcus*, *Propioniferax* and *Tessaracoccus*. The cells of the novel strains were Gram-positive, non-motile, non-spore-forming pleomorphic rods. They produced catalase but not oxidase, and nitrate reduction did not occur in peptone/yeast extract/glucose medium. Propionate and acetate were the predominant products of glucose fermentation. Fermentation occurred in the presence of 1,2-dichloroethane and 1,1,2-trichloroethane at concentrations up to at least 9.8 mM. The genomic DNA G + C content was 67.5–67.9 mol%. Menaquinone MK-9(H₄) was the predominant respiratory quinone and *meso*-diaminopimelic acid was present in the cell-wall peptidoglycan layer. The major cellular fatty acids were C_{15:0} and anteiso-C_{15:0}. On the basis of the results obtained in this study, strains BL-34^T and BL-35 should be classified within a novel taxon, for which the name *Brooklawnia cerclae* gen. nov., sp. nov. is proposed. The type strain of *Brooklawnia cerclae* is BL-34^T (=LMG 23248^T=NRRL B-41418^T). An additional strain, BL-35 (=LMG 23249=NRRL B-41419), was also characterized.

From 1969 until 1980, petrochemical wastes were disposed of by direct discharge to earthen ponds at the Brooklawn site, one of two areas that comprise what is now known as the Petro-Processors of Louisiana, Inc. Superfund Site, located near Baton Rouge (LA, USA). Although portions of the Brooklawn area were capped in the early 1990s and an array of wells was installed to recover contaminants, chlorosolvents remain in the subsurface both as soluble constituents within the groundwater and as non-aqueous-phase liquid (Clement *et al.*, 2002; US Environmental Protection Agency, 2005). The contaminants include 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, 1,2-dichloroethane, 1,2-dichloropropane, hexachloro-1,3-butadiene, hexachlorobenzene, vinyl

chloride and polycyclic aromatic hydrocarbons (Clement *et al.*, 2002; US Environmental Protection Agency, 2005). During a study aimed at characterizing the microbial population present within the non-aqueous-phase liquid source zone at the Brooklawn site, two novel bacterial strains, subsequently designated BL-34^T and BL-35, were isolated from chlorosolvent-contaminated anaerobic groundwater collected from well W-1024-1 of the site.

Strain BL-34^T was isolated on nutrient agar (Difco), supplemented with L-cysteine (0.5 g l⁻¹) as a reductant and resazurin (1.0 mg l⁻¹) as a redox indicator, adjusted to pH 7.0 prior to autoclaving. Incubation was at 30 °C in an anaerobic chamber (COY Instruments) containing a gas mixture comprising H₂/CO₂/N₂ (10:10:80, by vol.). Strain BL-35 was isolated on plate-count agar (Difco) with the same supplements and incubated under the same conditions. The purity of each strain was verified by microscopy after

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain BL-34^T is DQ196625.

multiple transfers. The strains were maintained on Colombia anaerobic sheep blood agar (CSBA) plates (BBL) or peptone/yeast extract/glucose (PYG) agar plates (Akasaka *et al.*, 2003). For comparison purposes, *Propionibacterium freudenreichii* NRRL B-3523^T was obtained from the Agricultural Research Service Culture Collection (Peoria, IL, USA).

Morphological features were observed using transmission electron microscopy (1000CX; JEOL) after negative staining with uranyl acetate (2%, v/v). Endospore production and motility were observed using phase-contrast microscopy (Optiphot; Nikon). Gram-staining, catalase, oxidase and nitrate-reduction tests were performed as described by Smibert & Krieg (1981). For the nitrate-reduction tests, cells were anaerobically incubated for 2 weeks at 30 °C in PYG medium amended with 10 mM sodium nitrate. Carbon-utilization tests were performed in 16 ml Hungate tubes containing 10 ml PY medium (Akasaka *et al.*, 2003) and a headspace of N₂/CO₂ (95:5, v/v). Each substrate was added at a concentration of 5 or 10 g l⁻¹ for monosaccharides, disaccharides and polysaccharides and sugar alcohols and at 15 or 30 mM for organic acids. Cultures were inoculated using 1% (v/v) exponentially growing seed culture grown in PYG. Cultures were recorded as positive if they exhibited an increase in optical density of more than 0.1 OD unit at 660 nm relative to controls (which lacked any added carbon sources). The pH range for growth in PYG was evaluated from pH 3.0 to 10.0, using the following buffers: 100 mM acetate buffer for pH 3.0–6.0; 100 mM potassium phosphate buffer for pH 6.0–8.0; and Tris buffer for pH 8.0–10.0. Growth with NaCl at 0–5% (w/v) and growth at 5–45 °C were determined in Hungate tubes containing 10 ml PYG. To determine whether vitamin B₁₂ stimulated growth of strains BL-34^T and BL-35, the cultures were grown in PYG medium with and without the addition of 10 µg vitamin B₁₂ l⁻¹.

The ability of the strains to use chlorosolvents as electron acceptors was tested in 25 ml serum bottles containing 10 ml anaerobic basal medium (Sung *et al.*, 2003) and headspace gas consisting of N₂/CO₂/H₂ (80:10:10, by vol.); the bottles were sealed with Teflon-lined butyl rubber stoppers and aluminium crimp seals. Basal medium in each bottle was amended with acetate, lactate and pyruvate (2 mM each) as potential carbon and energy sources and one of the following chlorosolvents as a potential electron acceptor (each 2.0 µmol): 1,1,2,2-tetrachloroethene, 1,1,2-trichloroethane, 1,2-dichloroethane, trichloroethene, *cis*-1,2-dichloroethene or 1,2-dichloropropane. Exponentially growing cells cultured in PYG broth were used as inocula. Uninoculated bottles served as negative controls. Bottles were incubated in the dark at 30 °C. The ability of the strains to degrade aromatic compounds under anaerobic conditions was tested using 25 ml serum bottles containing 10 ml anaerobic basal medium (Sung *et al.*, 2003) amended with 0.5 µmol benzene, toluene, ethylbenzene or *p*-xylene. The concentrations of the chlorosolvents and aromatic compounds were measured using a gas

chromatograph (HP6890; Hewlett Packard) equipped with a capillary column (60 m × 0.32 mm i.d., J&W P/N 113-4362; GS-Gaspro) and a flame-ionization detector.

The ability of strains BL-34^T and BL-35 to grow in the presence of various concentrations (0–9.8 mM) of 1,2-dichloroethane or 1,1,2-trichloroethane was tested in 120 ml serum bottles containing 80 ml PYG supplemented accordingly. The PYG used for strain BL-35 was also supplemented with 10 µg vitamin B₁₂ l⁻¹. The gas headspace mixture was N₂/CO₂ (95:5, v/v).

The fermentation products from cultures grown anaerobically in PYG medium were analysed by ion chromatography using Metrohm peak 761 compact apparatus equipped with a Metrosep organic acid column (25 cm × 7.8 mm) and a conductivity detector. Isocratic elution was performed with 0.5 mM H₂SO₄ at a flow rate of 0.5 ml min⁻¹ and at a constant temperature of 25 °C.

Extraction of genomic DNA, PCR amplification and sequencing of 16 rRNA genes were carried out as described by Rainey *et al.* (1996). Purified sequencing-reaction products were electrophoresed using a model 3100 DNA sequencer (Applied Biosystems). The 16S rRNA gene sequences of strains BL-34^T and BL-35 were aligned against previously determined actinobacterial sequences from the database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) using BioEdit, version 4.7.8 (Hall, 1999) and CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic analyses were performed using ARB (Strunk & Ludwig, 1995; <http://www.arb-home.de/>). The neighbour-joining algorithm was used to construct the phylogenetic tree from distance matrices calculated with the Jukes–Cantor correction (Jukes & Cantor, 1969). A bootstrap analysis was performed using PHYLIP, version 3.62 (Felsenstein, 2004; <http://evolution.genetics.washington.edu/phylip.html>), with 1000 resamplings.

Cells for the analysis of fatty acids were harvested from cultures grown at 30 °C in PYG under anaerobic conditions for 3 days following inoculation with a 1% (v/v) volume of exponentially growing culture. Fatty acids were extracted as described by White *et al.* (1979). The total lipid extract was fractionated by using silicic acid chromatography, and the polar fraction was transesterified to fatty acid methyl esters and analysed by gas chromatography/mass spectrometry (Guckert *et al.*, 1985). Menaquinones were extracted and analysed via HPLC by the Identification Services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) using the method described by Tindall (1990a, b). The occurrence of diaminopimelic acid in the cell wall and the peptidoglycan type were analysed by the Identification Services of the DSMZ, as described by Schleifer & Kandler (1972), using TLC on cellulose plates with the solvent system of Rhuland *et al.* (1955). The genomic DNA G+C content was determined by HPLC as described by Mesbah *et al.* (1989) following DNA isolation as described by Nielsen *et al.* (1995).

After 13 days anaerobic incubation on CSBA plates at 30 °C, strains BL-34^T and BL-35 formed white, circular, convex, smooth-surfaced colonies that were 1–2 mm in diameter. Better growth was observed on PYG agar: the colonies were identical in morphology to those grown on CSBA except that they were larger in size (2–3 mm in diameter) after a shorter period of incubation (4 days). No spore formation or motility was observed. The cells exhibited pleomorphic rod-type morphologies and were Gram-positive, catalase-positive and oxidase-negative. No nitrate reduction was observed. Growth occurred on nutrient agar, PYG agar and R2A agar (Difco) under both aerobic and anaerobic conditions; however, better growth was obtained under anaerobic conditions. Propionate and acetate were the predominant fermentation products detected in PYG cultures. The temperature range for growth was 10–40 °C, with an optimum of 37 °C. The pH range for growth was 4.5–8.0, with optimal growth at pH 6.5. Growth was sustained in the presence of NaCl at concentrations ranging from 0 to 3 % (w/v).

The results of additional physiological tests are given in the species description and in Table 1. Strains BL-34^T and BL-35 exhibited identical features except for the fact that the latter required vitamin B₁₂ supplementation for optimal growth in PYG under anaerobic conditions. No degradation of chlorosolvents (1,1,2-trichloroethane, 1,2-dichloroethane, 1,1,2,2-tetrachloroethane, trichloroethene, *cis*-1,2-dichloroethene, 1,2-dichloropropane) or aromatic hydrocarbons (benzene, toluene, ethyl benzene, *p*-xylene) was detected under anaerobic conditions, even after incubation for 3 months.

Although strains BL-34^T and BL-35 were unable to biotransform the chlorosolvents tested in this study, both grew well in PYG media in the presence of 1,2-dichloroethane and 1,1,2-trichloroethane at concentrations up to at least 9.8 mM. The fermentation products produced by strains BL-34^T and BL-35 in the presence of high chlorosolvent concentrations were the same as those produced in the absence of chlorosolvents. The fact that the strains are able to grow even in the presence of very high chlorosolvent concentrations probably explains why these micro-organisms are able to survive in the dense non-aqueous phase liquid (DNAPL) source zone from which they were isolated.

The major cellular fatty acids in both isolates were anteiso-C_{15:0} (51.9 % for BL-34^T and 61.4 % for BL-35) and C_{15:0} (33.2 % for BL-34^T and 24.7 % for BL-35). Minor cellular fatty acids included C_{14:0}, iso-C_{14:0}, C_{15:1}, C_{16:0}, iso-C_{16:0}, C_{16:1}ω7c, C_{17:0}, iso-C_{17:0} and anteiso-C_{17:0} (Table 1). The predominant respiratory quinone was MK-9(H₄). The peptidoglycan type was A1γ, with *meso*-diaminopimelic acid (*meso*-DAP) in the cell wall. The genomic DNA G+C contents of BL-34^T and BL-35 were 67.5 and 67.9 mol%, respectively.

A neighbour-joining tree constructed on the basis of comparison of 1363 nucleotide positions showed that strains BL-34^T and BL-35 belong to the family *Propionibacteriaceae*,

clustering with *Propionimicrobium lymphophilum* DSM 4903^T (Stackebrandt *et al.*, 2002) and uncultured bacterial clones N40 and GLY2B01 derived from anaerobic bioreactors (GenBank accession numbers AB195901 and AB24-4748) (Fig. 1). Environmental 16S rRNA clones N40 and GLY2B01 show the highest levels of sequence similarity with strains BL-34^T and BL-35 (95.7–96.0 %). Strains BL-34^T and BL-35 had identical 16S rRNA gene sequences over the 1459 nucleotide positions determined in this study.

Strains BL-34^T and BL-35 are most closely related to species of the genera *Propionimicrobium*, *Propionibacterium*, *Luteococcus*, *Propioniferax* and *Tessaracoccus* within the family *Propionibacteriaceae*. The type strain of *Propionimicrobium lymphophilum* (Stackebrandt *et al.*, 2002), the only species of the genus, clusters together with BL-34^T and BL-35 in the phylogenetic analysis and shows 93.5 % sequence similarity (Fig. 1). Members of the genus *Propionimicrobium* are clearly distinguishable from strains BL-34^T and BL-35 by their chemotaxonomic properties, i.e. by the presence of lysine rather than *meso*-DAP in the cell wall, by having C_{18:1}ω9c (30 %) as the predominant cellular fatty acid and by having a lower DNA G+C content (53–56 mol%) (Table 1).

The genus *Propionibacterium* comprises 11 species with validly published names. The phylogenetic position of four of these species is shown in Fig. 1, and strains BL-34^T and BL-35 are clearly outside the radiation of this genus. Although most of the species contain LL-DAP, a feature that clearly distinguishes them from strains BL-34^T and BL-35, it was reported that the type species, *Propionibacterium freudenreichii*, and some strains of *Propionibacterium acnes* and *Propionibacterium avidum* contain *meso*-DAP (Cummins & Johnson, 1986). Strains BL-34^T and BL-35, however, can be differentiated from the type species of the genus *Propionibacterium* by their cellular fatty acid composition (the presence of C_{18:1}, C_{21:0} and C_{22:0} as well as the markedly different content of iso-C_{14:0}, anteiso-C_{15:0} and iso-C_{16:0} in the latter) (Table 1). Additionally, no species of the genus *Propionibacterium* show more than 94 % 16S rRNA gene sequence similarity (Fig. 1) to strains BL-34^T and BL-35, suggesting that strains BL-34^T and BL-35 are distantly related to them and should be classified within a different genus. Additional distinguishing characteristics of the type species *Propionibacterium freudenreichii*, as well as the more closely related *Propionibacterium propionicum*, are listed in Table 1.

The genus *Luteococcus* currently contains three species with validly published names, namely *Luteococcus japonicus* Tamura *et al.* 1994 (the type species), *L. sanguinis* Collins *et al.* 2003 and *L. peritonei* Collins *et al.* 2000, which represent a distinct phylogenetic branch unrelated to strains BL-34^T and BL-35 (Fig. 1). They are also easily distinguished from strains BL-34^T and BL-35 by the chemotaxonomic features of diaminopimelic acid and the cellular fatty acids. Specifically, all *Luteococcus* species contain LL-DAP, and the predominant fatty acids of the type species, *L. japonicus*,

Table 1. Characteristics of strains BL-34^T and BL-35 and related taxa

Taxa: 1, strain BL-34^T (data from this study); 2, strain BL-35 (this study); 3, *Propionimicrobium lymphophilum* (Stackebrandt *et al.*, 2002); 4, *Propionibacterium freudenreichii* (Cummins & Johnson, 1986; Kusano *et al.*, 1997; this study) (the fatty acid composition of strain NRRL B-3523^T was determined in this study following cell growth under the same conditions and using the same analytical techniques as for strains BL-34^T and BL-35); 5, *Propionibacterium propionicum* (Cummins & Moss, 1990; Maszenan *et al.*, 1999); 6, *Luteococcus japonicus* (Tamura *et al.*, 1994; Maszenan *et al.*, 1999); 7, *Tessarcoccus bendigoensis* (Maszenan *et al.*, 1999); 8, *Propioniferax innocua* (Pitcher & Collins, 1991; Yokota *et al.*, 1994; Schumann *et al.*, 1997; Maszenan *et al.*, 1999). All isolates are Gram-positive, non-motile, non-spore-forming chemo-organotrophs. Symbols: +, positive test result; −, negative test result; V, variable; ND, not determined.

[illegible]

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8
C _{17:0}	3.2	1.1	—	0.9	—	3.1	—	—
iso-C _{17:0}	0.2	0.2	—	1.1	—	—	—	2.2
anteiso-C _{17:0}	0.6	0.8	2.5	5.3	—	—	—	6.7
C _{17:1}	—	—	—	—	—	29.6	—	—
iso-C _{17:1}	—	—	—	—	—	—	8.1	1.3
anteiso-C _{17:1}	—	—	—	—	—	—	—	2.9
C _{18:0}	—	—	—	11.1	—	1.8	—	—
C _{18:1}	—	—	—	—	—	15.4	—	—
iso-C _{18:0}	—	—	—	5.2	—	—	—	—
C _{18:1ω7c}	—	—	2.5	0.1	—	—	—	—
C _{18:1ω8c}	—	—	—	—	2–22	—	—	—
C _{18:1ω9c}	—	—	30	0.6	2–5	—	—	—
C _{21:0}	—	—	—	4.7	—	—	—	—
C _{22:0}	—	—	—	3.0	—	—	—	—

*Values are percentages of total fatty acids. In the case of data taken from other studies, dashes indicate fatty acids that were not reported.

are C_{16:0} and C_{17:0}, whereas strains BL-34^T and BL-35 contain anteiso-C_{15:0} and C_{15:0}. *Tessaracoccus bendigoensis* (Maszenan *et al.*, 1999) and *Propioniferax innocua* (Yokota *et al.*, 1994), also distantly related to BL-34^T and BL-35, are differentiated from these two strains in containing LL-DAP. *T. bendigoensis* also contains two menaquinones, MK-9(H₄) and MK-7(H₄), while strains BL-34^T and BL-35 possess only MK-9(H₄). Other distinguishing characteristics are summarized in Table 1.

On the basis of the phylogenetic, chemotaxonomic and phenotypic features obtained in this study, strains BL-34^T and BL-35 are clearly distinct from other genera in the family *Propionibacteriaceae*. We propose that strains BL-34^T and BL-35 be placed within a novel genus, *Brooklawnia* gen. nov., with *Brooklawnia cerclae* sp. nov. as the type species.

Description of *Brooklawnia* gen. nov. Rainey, da Costa and Moe

Brooklawnia (Brook.law'ni.a. N.L. fem. n. *Brooklawnia* named after Brooklawn, the contaminated site from which members of the genus were first isolated).

Cells are Gram-positive, non-motile, non-spore-forming pleomorphic rods. Mesophilic, facultatively anaerobic and chemoheterotrophic. Cells are catalase-positive and oxidase-negative, and nitrate is not reduced in PYG media. Propionate and acetate are the predominant products of glucose fermentation. Cell-wall peptidoglycan contains *meso*-DAP. Major cellular fatty acids are anteiso-C_{15:0} and C_{15:0}. MK-9(H₄) is the predominant respiratory quinone. Genomic DNA G + C content is 67.5–67.9 mol%. On the basis of 16S rRNA gene sequences, the genus belongs to the family *Propionibacteriaceae*. The type species is *Brooklawnia cerclae*.

Description of *Brooklawnia cerclae* sp. nov. Rainey, da Costa and Moe

Brooklawnia cerclae (cer.cla'e. N.L. gen. fem. n. *cerclae* of CERCLA, an arbitrary name formed from CERCLA, the acronym for the Comprehensive Environmental Response, Compensation, and Liability Act, which has mandated the clean-up of many hazardous waste sites in the USA).

Displays the following properties in addition to those given in the genus description. Growth occurs between 10 and 40 °C; optimum growth temperature is about 37 °C. Growth occurs between pH 4.5 and pH 8.0; optimum pH for growth is 6.5. Growth is not stimulated by the addition of NaCl, but is sustained in the presence of up to 3 % NaCl (v/v). Facultative anaerobic growth is supported by fermentation. Cells grow on arabinose, fructose, glucose, galactose, maltose, rhamnose, xylose, ribose, mannose, starch, glycogen, glycerol, mannitol, lactate and pyruvate, but not on acetate, adonitol, cellobiose, cellulose, dulcitol, erythritol, ethanol, fucose, fumarate, inositol, lactose, malate, methanol, raffinose, sorbitol, succinate, sucrose or xylan.

Fig. 1. Phylogenetic relationship of strains BL-34^T and BL-35 to species of the family Propionibacteriaceae based on 16S rRNA gene sequences, generated by the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 resamplings) are shown at branch points. Bar, 1 substitution per 100 nucleotide positions.

The type strain is strain BL-34^T (= LMG 23248^T = NRRL B-41418^T). An additional strain of the species is represented by BL-35 (= LMG 23249 = NRRL B-41419).

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