Clostridium chromiireducens sp. nov., isolated from Cr(VI)-contaminated soil

K. S. Inglett,¹ H. S. Bae,¹ H. C. Aldrich,^{2†} K. Hatfield³ and A. V. Ogram^{1, 2}

Correspondence

A. V. Ogram
aogram@ufl.edu

¹Soil and Water Science Department, University of Florida, Gainesville, FL 32611, USA

²Microbiology and Cell Science Department, University of Florida, Gainesville, FL 32611, USA

³Civil and Coastal Engineering Department, University of Florida, Gainesville, FL 32611, USA

A Cr(VI)-resistant, Gram-positive, spore-forming, obligate anaerobe, designated GCAF-1^T, was isolated from chromium-contaminated soil by its ability to reduce Cr(VI) in low concentrations. Mixed acid fermentation during growth on glucose resulted in accumulation of acetate, butyrate, formate and lactate. Morphological studies indicated the presence of peritrichous flagella, pili and an S-layer. The major cellular fatty acids (>5%) were C_{16:0}, C_{14:0}, summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), C_{18:1}ω7c, C_{16:1}ω9c, summed feature 4 (comprising iso-C_{17:1} I and/or anteiso-C_{17:1} B) and C_{18:1}ω9c. The DNA G + C content of strain GCAF-1^T was 30.7 mol%. Phylogenetic interference indicated that strain GCAF-1^T clustered with group I of the genus *Clostridium*. Of strains within this cluster, strain GCAF-1^T shared the highest 16S rRNA gene sequence similarities (98.1–98.9%) with *Clostridium beijerinckii* DSM 791^T, *C. saccharobutylicum* NCP 262^T, *C. saccharoperbutylacetonicum* N1-4^T, *C. puniceum* DSM 2619^T and *C. roseum* DSM 51^T. However, strain GCAF-1^T could be clearly distinguished from its closest phylogenetic neighbours by low levels of DNA–DNA relatedness (<50%) and some phenotypic features. Based on the evidence presented here, strain GCAF-1^T (=DSM 23318^T =KCTC 5935^T) represents a novel species of the genus *Clostridium*, for which the name *Clostridium chromiireducens* sp. nov. is proposed.

In recent decades, microbial metal reduction has been identified as an important process for the mineralization of organic compounds (Lovley & Phillips, 1986; Lovley, 1995) and the remediation of soils contaminated with toxic metals (Anderson & Lovley, 1997; Lovley & Coates, 1997). Fermentative bacteria play an important role in the decomposition of organic matter in anaerobic soils, but their potential role in metal reduction in these soils has not been widely studied. The genus *Clostridium* forms one of the largest genera of Gram-positive bacteria and its members are found in most anaerobic environments. Given the widespread distribution of members of the genus *Clostridium* in the environment, identifying metal reducing members of the genus may help to elucidate specific pathways for metal reduction in anaerobic soils and sediments, particularly those with high inputs of organic matter that are conducive to fermentation.

This study reports the characterization of a previously undescribed strain, designated GCAF-1^T, which is capable

of Fe(III) reduction via electron shuttles. Strain GCAF-1^T was isolated from a Superfund site in the Upper Peninsula of Michigan. The site is a wetland that received Cr(VI)-rich effluents from an adjacent leather-tanning facility for several years.

Anaerobic enrichments were prepared in bicarbonate-buffered liquid basal medium, as described by Lovley & Phillips (1988), supplemented with 10 mM glucose as the carbon and energy source, 400 μM Cr(VI), 5 mM Fe(OH)₃ and 100 μM of the humic acid analogue anthraquinone 2,6-disulfonate (AQDS; Sigma) as the electron acceptors. Enrichments were initiated with 10 g soil and conducted in the dark at 30 °C without shaking. Positive Cr(VI)-reducing enrichments were visually apparent by observing the loss of yellow colour due to reduction of Cr(VI), and the reduction of Cr(VI) was confirmed by chemical analyses (Urone, 1955). Positive enrichments were transferred to fresh medium (10% inoculum) four times. Strain GCAF-1^T was isolated from distinct colonies observed in an enriched mixed culture, grown using the roll tube method (Hungate, 1969). The colonies were suspended in fresh medium and subcultured when the medium was turbid with growth. Once purified, strain GCAF-1^T was routinely cultivated on basal medium supplemented with glucose, 400 μM Cr(VI), 5 mM Fe(OH)₃ and 100 μM AQDS.

†Deceased.

Abbreviation: AQDS, 2,6-anthraquinone disulfonate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GCAF-1^T is AY228334.

Microscopic examinations were performed on cells harvested from overnight anaerobic cultures grown in basal medium supplemented with glucose at 30 °C. Motility was observed by phase-contrast microscopy. For scanning electron microscopy, cells were fixed at room temperature for 2 h in 2.5 % glutaraldehyde with 0.1 M sodium cacodylate buffer (pH 7.2). After a brief wash in distilled water, the samples were dehydrated in 75 % ethanol and 100 % ethanol for 10 min each and in hexamethyl disilazane for 15 min and then air-dried on aluminium stubs. After sputter coating with gold, samples were viewed and photographed using a Hitachi S-570 scanning electron microscope and a custom-fitted 35 mm camera. For the preparation of thin sections, cells were fixed in 2.5 % glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) at room temperature for 30 min. After two buffer washes, the cells were post-fixed in 1 % osmium tetroxide at 4 °C for 30 min and then washed twice in distilled water. The cells were then dehydrated through an alcohol series, after which they were stained with 1 % uranyl acetate in 75 % ethanol for 1 h. The cells were then embedded in Spurr's low viscosity resin, sectioned with an LKB Nova ultramicrotome, post-stained with lead citrate and examined and photographed with a Zeiss EM-10CA transmission electron microscope. For negative staining, a drop of cell suspension was placed on a Formvar-coated copper grid, which was placed on distilled water for 1 minute and then floated on 1 % aqueous uranyl acetate for 1 minute. After drying, grids were examined and photographed using TEM at 100 kV. The periodic acid silver hexamine stain for polysaccharides was performed as described by Aldrich (1974).

For the determination of phenotypic properties, test cultures were incubated anaerobically for up to 48 h at 30 °C, unless indicated otherwise. Reduction of Cr(VI) was tested with 50 ml basal medium supplemented with glucose, Cr(VI) and *s*-diphenylcarbazide to assay Cr(VI), as described by Urone (1955). For substrate utilization studies, 10 mM of each electron donor (lactate, formate, acetate, citrate, butyrate and benzoate) was added separately from sterile stock concentrations to basal medium supplemented with 400 µM Cr(VI), 5 mM Fe(OH)₃ and 100 µM AQDS. Cells were enumerated by epifluorescent microscopy (Hobbie *et al.*, 1977). Growth was regarded as positive if the resultant turbidity was greater than that observed in tubes with no electron donor. The API 20A identification kit (bioMérieux) was used to determine some phenotypic features, according to the manufacturer's recommended protocols, using cells grown on Peptone–yeast extract–glucose (PYG; Akasaka *et al.*, 2003) agar for 3 days. The ability of strain GCAF-1^T to grow aerobically was tested at 30 °C in aerobically prepared basal medium supplemented with 10 mM glucose. Strain GCAF-1^T was shown not to produce riboflavin in medium supplemented with whole milk under anaerobic conditions (Keis *et al.*, 2001), which distinguished it from *Clostridium acetobutylicum*. Organic acids that accumulated as fermentation products were determined from anaerobic cultures in PYG broth after incubation for 3 days at 28 °C using HPLC (Waters) equipped with a UV detector, as described by Albert & Martens (1997).

For the extraction of fatty acids, cells were harvested after anaerobic growth on PYG agar for 3 days. Fatty acid methyl esters were prepared according to Metcalfe *et al.* (1966) and analysed using the Microbial Identification

Fig. 1. Electron micrographs of cells of strain GCAF-1^T. (a) Late exponential-phase cells. (b) Cells showing silver-stained glycogen inclusions (G). (c) Ultrathin cell section showing the S-layer (SL); outer membrane (OM), cytoplasmic membrane (CM) and spore (Sp) are also indicated. (d) Negatively stained cell showing flagella (F) and pili (P). Bars, 12 µm (a).

System (MIDI). After digesting extracted DNA with P1 nuclease (Mesbah *et al.*, 1989), the base composition of the DNA was determined at the DSMZ by HPLC, as described by Tamaoka & Komagata (1984).

16S rRNA gene sequencing and phylogenetic analysis were conducted as described previously (Lane, 1991; Uz & Ogram, 2006). Sequencing was performed by the Interdisciplinary Center for Biotechnology Research core sequencing facility of the University of Florida. The phylogenetic analysis included sequences obtained from comparative searches of public databases and was conducted using the neighbour-joining method (Saitou & Nei, 1987) with Jukes–Cantor correction (Jukes & Cantor, 1969) in MEGA version 4 (Tamura *et al.*, 2007), followed by a bootstrap analysis using a heuristic search strategy and 1000 replications (Felsenstein, 1985).

DNA–DNA hybridizations between strain GCAF-1^T and *Clostridium beijerinckii* DSM 791^T, *Clostridium saccharobutylicum* DSM 13864^T, *Clostridium puniceum* DSM 2619^T and *Clostridium roseum* DSM 7320^T were performed at the DSMZ using a model Cary 100 Bio UV/VIS spectrophotometer, as described by De Ley *et al.* (1970), under consideration of the modification described by Huß *et al.* (1983). Additionally, DNA–DNA relatedness between strain GCAF-1^T and *Clostridium saccharoperbutylacetonicum* DSM 14923^T was estimated at the Korean Collection for Type Cultures using a fluorometric method with photobiotin-labelled DNA probes and microdilution wells, according to Ezaki *et al.* (1989).

Strain GCAF-1^T was a spore-forming obligate anaerobe that grew well on basal medium with glucose as the electron donor. Colonies were white, translucent and convex with smooth edges, which became irregular after 2 days. Cells were rods (4–7 × 1–1.5 µm) and occurred singly (Fig. 1a) or in chains of between four and seven cells (data not shown). Scanning electron microscopy revealed oval terminal and subterminal endospores (1–1.5 × 0.5 µm) in cultures grown in the presence of glucose for 24 h. In the presence of 400 µM Cr(VI), spore formation was not observed within 24 h. Cells of strain GCAF-1^T were motile, as seen under electron microscopy with a freshly drawn sample. Electron-lucent inclusions were noted in the cytoplasm of stationary-phase cells and were suspected to be glycogen (Fig. 1b). Positive results with periodic acid silver hexamine staining were observed. Electron-dense silver was noted in the cell-wall lipopolysaccharide and in the electron-lucent cytoplasmic inclusions, strongly suggesting that these inclusions contained glycogen. An ultrathin section of strain GCAF-1^T showed the presence of an S-layer (Fig. 1c). S-layers, or surface layers, are the outermost components of the cell walls of several bacterial and archaeal species (Beveridge, 1994; Lemaire *et al.*, 1998) and are known to function as molecular sieves, protective coats and adhesion sites for exoenzymes (Sára, 2001). Negative staining of strain GCAF-1^T revealed the presence of peritrichous flagella (Fig. 1d) and pili, such as those

observed for other members of the genus *Clostridium* (Holt *et al.*, 1994).

In the presence of Fe(III) and AQDS, strain GCAF-1^T completely reduced 400 µM Cr(VI). In the absence of Fe(III) and AQDS, complete reduction of Cr(VI) was observed only when Cr(VI) was present in low concentrations (20 µM). Reduction of Cr(VI) occurred simultaneously with the growth of strain GCAF-1^T and no reduction was observed in cell-free controls. The generation time of strain GCAF-1^T in the absence of Cr(VI) was 2.0 h. In the presence of 15, 35 and 50 µM Cr(VI), the generation times were 2.5, 4.0 and 4.5 h, respectively. The primary

Table 1. Cellular fatty acid compositions of strain GCAF-1^T and closely related phylogenetic neighbours in the genus *Clostridium*

Strains: 1, GCAF-1^T; 2, *C. saccharoperbutylacetonicum* DSM 14923^T; 3, *C. puniceum* DSM 2619^T; 4, *C. saccharobutylicum* DSM 13864^T; 5, *C. beijerinckii* DSM 791^T; 6, *C. roseum* DSM 7320^T. Data were taken from this study. Major fatty acids (>5.0%) are shown in bold. Fatty acids representing <0.5% of the total are not shown. ECL, Equivalent chain-length.

Fatty acid (%)	1	2	3	4	5	6
C _{12:0}	1.47	—	—	0.97	1.17	—
C _{14:0}	9.86	5.19	1.91	13.72	3.66	2.17
iso-C _{14:1}	—	—	0.60	—	—	—
C _{16:0}	45.81	32.02	36.74	47.16	44.09	47.26
C _{16:0} N alcohol	—	1.13	3.07	—	1.12	12.51
C _{16:1ω5c}	—	—	—	—	—	0.67
C _{16:1ω9c}	7.63	9.98	11.01	1.67	—	1.03
C _{16:1ω9c}	—	—	—	—	7.61	—
iso-C _{16:1}	—	—	—	—	—	0.56
C _{17:0} cyclo	—	9.42	—	6.00	8.37	1.02
C _{17:0} 10-methyl	—	—	—	—	—	1.13
iso-C _{17:1ω5c}	—	—	—	—	—	2.72
C _{18:1ω7c}	9.02	2.80	6.00	6.43	0.72	2.85
C _{18:1ω9c}	5.49	1.16	2.51	0.71	0.97	1.07
C _{18:0}	2.74	1.34	1.29	1.12	1.58	1.43
iso-C _{19:0}	—	1.25	—	—	—	—
C _{19:0} cyclo ω8c	—	4.10	—	1.24	3.77	13.52
C _{19:0} 10 methyl	—	2.23	—	1.37	1.96	7.23
Summed features*						
1	—	1.04	—	—	—	—
3	9.40	7.70	14.18	11.97	5.11	0.85
4	5.90	16.76	16.71	5.69	15.90	—
7	—	—	—	—	—	3.59
Unknown ECL	0.91	3.32	3.82	—	3.11	—
14.96						

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 1 comprises iso-C_{15:1} H and/or C_{13:0} 3-OH. Summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1ω7c}. Summed feature 4 comprises iso-C_{17:1} I and/or anteiso-C_{17:1} B. Summed feature 7 comprises unknown ECL 18.846 and/or C_{19:1ω6c}.

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships between strain GCAF-1^T and members of the genus *Clostridium*. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

fermentation products from glucose in PYG medium were acetate, lactate, formate and butyrate, which indicated a mixed acid fermentation.

The most prevalent fatty acids of strain GCAF-1^T were C_{16:0} (45.8%), C_{14:0} (9.9%), summed feature 3 (comprising iso-C_{15:0}, 2-OH and/or C_{16:1}ω7c; 9.4%), C_{18:1}ω7c (9.0%), C_{16:1}ω9c (7.6%), summed feature 4 (comprising iso-C_{17:1} I and/or anteiso-C_{17:1} B; 5.9%) and C_{18:1}ω9c (5.5%)

(Table 1). The fatty acid methyl ester profiles of type strains of species of the genus *Clostridium* with high (>98.0%) 16S rRNA gene sequence similarity to strain GCAF-1^T were analysed after cultivation under similar conditions. All strains contained C_{16:0} as the most dominant fatty acid (32–47%); however, proportions of the other major fatty acids differed among strains (Table 1). The G+C content of DNA of strain GCAF-1^T was 30.7 mol%, which is within the range reported for the genus *Clostridium* (21–54 mol%).

Table 2. Phenotypic characteristics of strain GCAF-1^T and closely related phylogenetic neighbours in the genus *Clostridium*

Strains: 1, GCAF-1^T; 2, *C. saccharoperbutylacetonicum* DSM 14923^T; 3, *C. puniceum* DSM 2619^T; 4, *C. saccharobutylicum* DSM 13864^T; 5, *C. beijerinckii* DSM 791^T; 6, *C. roseum* DSM 7320^T. Data were obtained from this study unless otherwise indicated. All strains were straight rods and motile with peritrichous flagella. All strains were negative for urease and indole production. +, Positive; –, negative; ND, no data available; A, acetate; B, butyrate; F, formate; L, lactate; P, propionate; S, succinate (lower-case letters indicate minor components <10% of total fermentation products).

Characteristic	1	2	3	4	5	6
Cell dimensions (μm)*						
Width	1.0–1.5	0.4–0.8 ^a	0.6 ^b	1.4–3.8 ^a	0.5–1.7 ^a	0.7–0.9 ^c
Length	4.0–7.0	3.1–6.1 ^a	1.8–4.2 ^b	6.3–10 ^a	1.7–8.0 ^a	3.3–4.3 ^c
Optimal growth (°C)*	30–35	25–35 ^a	23–33 ^b	30–34 ^a	37 ^a	37 ^c
DNA G+C (mol%)*	31	31 ^a	28–29 ^b	28–32 ^a	26–28 ^a	ND ^c
Fermentation products	A, F, B, l	A, F, L, b	A, F, L, B, S	A, F, L, B, s	F, L, B, S, a, p	A, F, L, B
Aesculin hydrolysis†	–	–	+	+	–	+
Acidification of: †						
Mannitol	+	+	–	–	–	–
Maltose	+	+	+	–	+	–
Glycerol	+	+	–	+	–	–
Cellobiose	+	+	+	–	+	+
Mannose	+	+	–	–	+	–
Melezitose	+	–	–	–	+	–
Raffinose	+	+	+	–	+	–
Sorbitol	+	–	–	–	–	–
Rhamnose	+	+	–	–	–	–
Trehalose	+	+	+	+	+	–
Glucose	+	+	–	–	–	–
Lactose	+	+	–	+	+	–
Saccharose	+	+	+	–	+	–
Salicin	+	+	–	–	+	–
Xylose	+	+	–	–	+	–
Arabinose	+	+	–	+	+	–

*Data taken from: *a*, Keis *et al.* (2001); *b*, Lund *et al.* (1981); *c*, Cato *et al.* (1986).

†Data taken from this study using API 20A with cells grown on PYG agar under anaerobic conditions for 3 days.

The phylogenetic analysis (Fig. 2) indicated that the 16S rRNA gene sequence of strain GCAF-1^T clustered within group I of the genus *Clostridium* (Collins *et al.*, 1994; Wiegel *et al.*, 2006). Within this cluster, strain GCAF-1^T was most closely related to *C. beijerinckii* DSM 791^T (98.9% 16S rRNA gene sequence similarity), *C. saccharobutylicum* NCP 262^T (98.1%), *C. saccharoperbutylacetonicum* N1-4^T (98.5%), *C. puniceum* DSM 2619^T (98.6%) and *C. roseum* DSM 51^T (98.8%). In cases with >97% 16S rRNA gene sequence similarity, DNA–DNA relatedness is critical in clarifying relationships between species at the generic level (Stackebrandt & Goebel, 1994). Strain GCAF-1^T exhibited <50% DNA–DNA relatedness with its closest phylogenetic neighbours: *C. beijerinckii* DSM 791^T, 23.5%; *C. saccharobutylicum* DSM 13864^T, 49.8%; *C. saccharoperbutylacetonicum* DSM 14923^T, 36.3%; *C. puniceum* DSM 2619^T, 33.9%; and *C. roseum* DSM 7320^T, 33.6%. These levels of DNA–DNA relatedness are low enough to classify strain GCAF-1^T in a species distinct from those previously described (Wayne *et al.*, 1987).

Several phenotypic characteristics of strain GCAF-1^T and its closest phylogenetic neighbours were compared (Table 2). Strain GCAF-1^T was distinct from the reference strains with regard to carbon utilization, morphological features, G + C content, fatty acid profile and other characteristics: no reference strain exhibited a combination of phenotypic characters identical to that of strain GCAF-1^T.

The distinct phenotypic and chemotaxonomic features and DNA–DNA relatedness supported the unique phylogenetic position of strain GCAF-1^T. Therefore, strain GCAF-1^T should be assigned as the type strain of a novel species in the genus *Clostridium*, for which the name *Clostridium chromiireducens* sp. nov. is proposed.

Description of *Clostridium chromiireducens* sp. nov.

Clostridium chromiireducens [chro.mi.i.re.du'cens. N.L. neut. n. *chromium* chromium; L. part. adj. *reducens* (from L. v. *reducere*) drawing backwards, bringing back to a state or condition; N.L. part. adj. *chromiireducens* chromium reducing].

Cells are obligately anaerobic, spore-forming, Gram-positive rods, $\sim 4.0\text{--}7.0 \times 1.0\text{--}1.5\ \mu\text{m}$. Cells are motile and exhibit peritrichous flagella. Colonies are white, convex and translucent, with smooth edges becoming irregular after incubation for 2 days. Glucose fermentation yields a mixed acid fermentation, with acetate, lactate, formate and butyrate accumulating in the medium. Cr(VI) and Fe(III) are reduced during growth on glucose. Resistant to rifampicin and capable of growth with glucose, mannitol, lactose, sucrose, maltose, salicin, xylose, arabinose, cellobiose, mannose, raffinose, trehalose, glycerol, melezitose, sorbitol, rhamnose and pectin. Does not produce urease and does not hydrolyse gelatin or aesculin. The major fatty acids (>5%) are C_{16:0}, C_{14:0}, summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), C_{18:1}ω7c,

C_{16:1}ω9c, summed feature 4 (comprising iso-C_{17:1} I and/or anteiso-C_{17:1} B) and C_{18:1}ω9c.

The type strain, GCAF-1^T (=DSM 23318^T =KCTC 5935^T), was isolated from a chromium-contaminated wetland soil. 16S rRNA gene sequence phylogeny places the type strain within group I of the genus *Clostridium*. The DNA G + C content of the type strain is 30.7 mol%.

Acknowledgements

This research was partially funded by the Natural and Accelerated Bioremediation Research (NABIR) program, Biological and Environmental Research (BER), US Department of Energy (grant no. DE-FG02-97ER62471) and the National Science Foundation (grant no. DEB-0841596). We thank Van Pham and Lorraine McDowell for technical assistance and Milind Chavan and Hector Castro for helpful discussions during preparation of the manuscript. We also thank Dr T. Marsh at Michigan State University for providing soil samples.

References

- Akasaka, H., Izawa, T., Ueki, K. & Ueki, A. (2003). Phylogeny of numerically abundant culturable anaerobic bacteria associated with degradation of rice plant residue in Japanese paddy field soil. *FEMS Microbiol Ecol* **43**, 149–161.
- Albert, D. B. & Martens, C. S. (1997). Determination of low-molecular weight organic acid concentrations in seawater and pore-water samples via HPLC. *Mar Chem* **56**, 27–37.
- Aldrich, H. C. (1974). Spore cleavage and the development of wall ornamentation in two myxomycetes. *Proc Iowa Acad Sci* **81**, 28–36.
- Anderson, R. T. & Lovley, D. R. (1997). Ecology and biogeochemistry of *in situ* groundwater bioremediation. *Adv Microb Ecol* **15**, 289–350.
- Beveridge, T. J. (1994). Bacterial S-layers. *Curr Opin Struct Biol* **4**, 204–212.
- Cato, E. P., George, W. L. & Finegold, S. M. (1986). Genus *Clostridium*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Hobbie, J. E., Daley, R. J. & Jasper, S. (1977). Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* **33**, 1225–1228.
- Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*, 9th edn. Baltimore, MD: Williams and Wilkins.

- Hungate, R. E. (1969).** A roll tube method for cultivation of strict anaerobes. In *Methods in Microbiology*, vol. 3B, pp. 117–132. Edited by R. Norris & D. W. Ribbons. New York: Academic Press.
- Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983).** Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jukes, T. H. & Cantor, C. R. (1969).** Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Keis, S., Shaheen, R. & Jones, D. T. (2001).** Emended descriptions of *Clostridium acetobutylicum* and *Clostridium beijerinckii*, and descriptions of *Clostridium saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov. *Int J Syst Evol Microbiol* **51**, 2095–2103.
- Lane, D. J. (1991).** 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.
- Lemaire, M., Miras, I., Gounon, P. & Béguin, P. (1998).** Identification of a region responsible for binding to the cell wall within the S-layer protein of *Clostridium thermocellum*. *Microbiology* **144**, 211–217.
- Lovley, D. R. (1995).** Bioremediation of organic and metal contaminants with dissimilatory metal reduction. *J Ind Microbiol* **14**, 85–93.
- Lovley, D. R. & Coates, J. D. (1997).** Bioremediation of metal contamination. *Curr Opin Biotechnol* **8**, 285–289.
- Lovley, D. R. & Phillips, E. J. P. (1986).** Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Appl Environ Microbiol* **51**, 683–689.
- Lovley, D. R. & Phillips, E. J. P. (1988).** Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl Environ Microbiol* **54**, 1472–1480.
- Lund, B. M., Brocklehurst, T. F. & Wyatt, G. M. (1981).** Characterization of strains of *Clostridium puniceum* sp. nov., a pink-pigmented, pectolytic bacterium. *J Gen Microbiol* **122**, 17–26.
- Mesbah, M., Premachandran, U. & Whitman, W. (1989).** Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Metcalfe, L. D., Schmitz, A. A. & Pelka, J. R. (1966).** Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal Chem* **38**, 514–515.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sára, M. (2001).** Conserved anchoring mechanisms between crystalline cell surface S-layer proteins and secondary cell wall polymers in Gram-positive bacteria? *Trends Microbiol* **9**, 47–49 (discussion 49–50).
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Urone, P. (1955).** Stability of colorimetric reagent for chromium, s-diphenylcarbazide, in various solvents. *Anal Chem* **27**, 1354–1355.
- Uz, I. & Ogram, A. V. (2006).** Cellulolytic and fermentative guilds in eutrophic soils of the Florida Everglades. *FEMS Microbiol Ecol* **57**, 396–408.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Wiegel, J., Tanner, R. & Rainey, F. A. (2006).** An introduction to the family *Clostridiaceae*. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 4, pp. 654–678. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.