NOTE

Reclassification of *Clostridium quercicolum* as *Dendrosporobacter quercicolus* gen. nov., comb. nov.Carsten Strömpl,¹ B. J. Tindall,³ Heinrich Lünsdorf,² Tit-Yee Wong,⁴ Edward R. B. Moore¹ and Hans Hippe³Author for correspondence: Carsten Strömpl. Tel: +49 531 6181 405. Fax: +49 531 6181 411.
e-mail: cst@gbf.de^{1,2} Microbial Ecology Group¹ and Electron Microscopy Unit², Division of Microbiology, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany³ DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124 Braunschweig, Germany⁴ Department of Microbiology and Molecular Cell Sciences, University of Memphis, Memphis, TN 38152, USA**Morphological features, genomic DNA base composition and 16S rDNA sequence similarities, as well as a distinct phospholipid pattern, whole-cell fatty acid distribution and the occurrence of the lipoquinone 'lipid F', indicate that *Clostridium quercicolum* belongs to the *Sporomusa–Pectinatus–Selenomonas* phyletic group and possesses only a remote relationship to members of the genus *Clostridium sensu stricto*. On the basis of these results, the new genus and combination *Dendrosporobacter quercicolus* gen. nov., comb. nov. are proposed.****Keywords:** *Dendrosporobacter* gen. nov., *Sporomusa–Pectinatus–Selenomonas* phyletic group, *Clostridium*

The broad definition of the genus *Clostridium* is based on a few key features and has allowed the assignment of a relatively wide range of physiologically, morphologically and biochemically heterogeneous organisms to this genus. This current definition requires only that organisms be obligately anaerobic, rod-shaped, spore-forming and non-sulfate-reducing and exhibit a Gram-positive-type cell wall (Cato *et al.*, 1986). Additional characteristics are necessary to separate these organisms taxonomically into discrete taxa (i.e. genera). Analyses of the 16S rRNA gene sequences of species of *Clostridium* and related genera have revealed that the genus *Clostridium sensu lato* is polyphyletic (Collins *et al.*, 1994) and related phylogenetically to several Gram-negative-staining, non-sporulating or coccoid taxa. These analyses have led to the recognition of several stable, distinct, genetic-based clusters. Some of these are consistent with metabolic features, while others will require further work in order to investigate congruence with the observed phenotypes and clarify their taxonomic status. During the course of unravelling the taxonomy of the clostridia, several species

have already been reclassified within newly created genera, such as *Oxobacter* or *Moorella*, or have been reclassified in existing genera such as *Eubacterium* (Collins *et al.*, 1994). One species that has remained misclassified is *Clostridium quercicolum* (Collins *et al.*, 1994).

C. quercicolum was isolated from discoloured tissue of living oak trees (Stankewich *et al.*, 1971) and is distinguishable from other clostridial species on the basis of differences in biochemical activities and genomic DNA base composition. The determination of the 16S rRNA sequence by C. R. Woese (unpublished data; see Maidak *et al.*, 1999) demonstrated that this organism is the only described species of *Clostridium* that clusters within the *Sporomusa–Pectinatus–Selenomonas* phyletic group. This phyletic group contains a heterogeneous assemblage of taxa commonly exhibiting a Gram-negative cell wall and a G+C content of genomic DNA significantly higher than that observed for members of *Clostridium* cluster I (i.e. *sensu stricto*). We undertook a polyphasic analysis, including electron microscopic and chemotaxonomic studies, in order to define further the relationship of *C. quercicolum* to the members of the *Sporomusa–Pectinatus–Selenomonas* phyletic group.

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Abbreviation: PY, peptone/yeast extract.

Fig. 1. Ultrastructure analysis of *C. quercicolum*. Morphology of negatively stained (a–d), ultrathin-sectioned (e, h–i) and shadow-cast (f–g, j) cells. Besides the normal symmetric insertion of the septation plane, cells often show unequal division (a–c; indicated by arrows). Vesicles (b; V) are seen as translucent structures. Elongated rod-shaped structures can be observed as distinct individual particles (b; double arrowhead) and their membrane-like character is obvious when seen in detail (d; arrowhead). Rod-shaped extrusions can be seen in contact with the cell surface (b; arrowheads) and from ultrathin sections it can be recognized that these extrusions are derived from the wavy outer membrane-like layer, outlined as a continuous double-track structure (e; arrowheads). One to three flagella (b, c, f; fl) are inserted in the lateral region of the cell body. As can be seen from a shadow-cast cell (f; open arrow indicates the shadowing direction), the flagellar insertion (f; open arrowheads) is subpolar. When seen in detail, the insertion is recognized by the hook origin in contact with the cell surface (g; double arrowhead). A single arrowhead indicates the transition of the hook into the flagellum (g; open arrowhead). In ultrathin sections, the translucent DNA (h; star) is homogeneously dispersed within the cytoplasm. Dividing cells show a deep septation cleft within the cell wall (h; arrow). Cell wall construction is Gram-negative, since a trilamellar, black-bright-black outer membrane-like layer (i; om) and a peptidoglycan continuum (i; mu) are present, adjacent to the cytoplasmic membrane (i; cm) at the cytoplasm/periplasm border. Rod-like protrusions, often organized in clusters, are associated with the cell (j; arrow). Bars represent 50 (d, e, g, i), 60 (j), 250 (a, f, h) and 600 (b, c) nm.

Morphology

C. quercicolum DSM 1736^T was grown anaerobically in peptone/yeast extract (PY) broth (Holdeman *et al.*, 1977) supplemented with 0.5% (w/v) fructose. The incubation temperature was 30 °C. The culture headspace contained N₂/CO₂ in a 1:1 ratio in Hungate tubes and a 4:1 ratio in large-scale cultures.

C. quercicolum cells in the mid-exponential phase of growth were used for electron microscopic studies. Negative-stain and ultrathin-section preparations were

carried out as described elsewhere (Yakimov *et al.*, 1998).

Cells for heavy metal shadow-casting were adsorbed to Formvar-coated Ni-grids (300 mesh) directly from the culture. After thorough blotting, the grids were air-dried and mounted for metal shadowing. Metal shadowing was carried out at an elevation of 15° by evaporation of Pt/C with an electron gun (MED 020; Baltec) for 15 s at a pressure of 5×10^{-6} mbar. Samples were observed in a Zeiss CEM 902 transmission

electron microscope at primary magnifications from $\times 7000$ to $\times 12000$.

In both negatively stained (Fig. 1a–c) and ultrathin-sectioned (Fig. 1h) samples of *C. quercicolum*, the cells were rod-shaped with lengths of 1.2–2.7 μm . However, the cell width ranged from 420 to 500 nm in negatively stained specimens and from 500 to 600 nm in ultrathin sections and shadowed samples. The micro-organism, although growing exponentially, demonstrated a tendency for unequal segmentation when dividing (Fig. 1a, b) and the septation cleft was rather narrow (Fig. 1a–c, h: arrows). In negatively stained cells and ultrathin sections, rod-shaped or vesicular structures could be recognized (Fig. 1a, b, d), which appeared to be derived from the outer membrane-like layer of the bacterium (Fig. 1e). Here, tubular extrusions occurred from the outer membrane-like layer. These tubular structures had an outer diameter of approximately 3–6 nm. In shadowed cells, similar tubular structures were organized in clusters on the cell surface (Fig. 1j). In this type of preparation, it could not be discerned whether they represented extrusions of the outer membrane-like surface or were some different kind of bacterial product.

In addition to the outer membrane-like surface, a distinct, centrally arranged, electron-dense layer could be recognized in ultrathin sections (Fig. 1i), which, morphologically, represents the peptidoglycan continuum. Considering all of these characteristic morphological details together, *C. quercicolum* possesses a distinct, Gram-negative cell-wall architecture. This agrees well with previous observations (Cato *et al.*, 1986; Stankewich *et al.*, 1971) that *C. quercicolum* stains uniformly Gram-negative.

It is evident from light microscopic observations that *C. quercicolum* is motile. Shadowed (Fig. 1f) and negatively stained samples (Fig. 1b, c) show one to three flagella associated with individual cells. Flagellum insertion, identified by its hook (Fig. 1f, g), was observed in the lateral region of the cell (Fig. 1f: arrowheads). Cells lost their flagella easily both in negatively stained and shadow-cast preparations.

Chemotaxonomy

Respiratory lipoquinones and polar lipids were extracted and separated from 100 mg freeze-dried cell material by the two-stage method described by Tindall (1990a, b). The major lipoquinones detected were 'lipid F' isoprenologues with eight (48%, relative percentage) and nine (43%) isoprenoid units attached to the naphthoquinone nucleus. 'Lipid F' with ten (7%) and with seven (2%) isoprenoid units were detected as minor compounds. A survey of the literature, together with the examination of species of the genus *Anaerovibrio*, suggests that 'lipid F' is characteristic of members of the *Sporomusa–Pectinatus–Selenomonas* phyletic group (Möller *et al.*, 1984; Schleifer *et al.*, 1990; Stackebrandt *et al.*, 1985;

Fig. 2. Two-dimensional TLC of the polar lipids of *C. quercicolum* DSM 1736^T. For details see Methods. PE, Phosphatidyl ethanolamine; PS, phosphatidyl serine; PN, amino-phospholipid; PL1, PL2, PL3, unidentified phospholipids; GL, unidentified glycolipid showing atypical staining behaviour; ?, material staining in a characteristic fashion with the anisaldehyde/sulfuric acid reagent but which could not be assigned a chemical structure.

Strömpl *et al.*, 1999), a finding that further supports the assignment of *C. quercicolum* to this evolutionary group. The polar lipids (Fig. 2) comprised two major phospholipids and one major glycolipid. The two phospholipids were identified as phosphatidyl ethanolamine and phosphatidyl serine, features which, together with a glycolipid of low R_f value, appear to delineate the members of the *Sporomusa–Pectinatus–Selenomonas* phyletic group (Van Golde *et al.*, 1973; Johnston & Goldfine, 1982; Kamio & Takahashi, 1980; Prins *et al.*, 1974; Shah *et al.*, 1983; Strömpl *et al.*, 1999; Verkley *et al.*, 1975; Watanabe *et al.*, 1982). While the major polar lipids indicated that *C. quercicolum* is a member of this evolutionary group, the presence of additional polar lipids enables further differentiation within this group, based on data currently available.

Fatty acids were analysed as the methyl ester derivatives, as described elsewhere (Strömpl *et al.*, 1999; B. J. Tindall, unpublished data). In contrast to previous reports of dimethyl acetals as components of the cells of members of the *Sporomusa–Pectinatus–Selenomonas* phyletic group (Moore *et al.*, 1994), these components were not detected. In contrast, significant levels of 3-hydroxy fatty acids were present (Table 1). Studies of members of the genus *Anaerovibrio* (Strömpl *et al.*, 1999) have shown that 3-hydroxy fatty acids may have been wrongly identified as dimethyl acetals in the work of Moore *et al.* (1994). The identity of the 3-hydroxy fatty acids was confirmed by GC-MS, as

Table 1. Fatty acid composition of *C. quercicolum* DSM 1736^T

Methods 1 and 2 refer to the differential release of fatty acids that indicate the presence of ester- and (presumptive) amide plus ester-linked fatty acids.

Fatty acid	Method 1 area (%)	Method 2 area (%)
10:0	1.3	1.0
11:0	8.9	7.4
?	—	1.3
3OH-11:0	3.8	3.8
?	0.7	1.3
3OH-12:0	1.5	1.6
?	0.7	2.1
14:0	1.5	1.3
?	—	0.6
3OH-13:0	2.3	10.2
15:1	28.3	24.0
15:1	0.7	0.6
15:0	10.5	9.2
16:1	5.2	4.5
16:1	2.0	1.8
16:0	1.8	1.6
17:1	3.5	3.2
17:1	23.0	20.7
17:1	0.8	0.7
17:0	1.9	1.9
18:1	1.7	1.5

?, Unknown structure; —, not detected.

described by Strömpl *et al.* (1999), and is consistent with their presence in other species in the *Sporomusa–Pectinatus–Selenomonas* phyletic group (Helander *et al.*, 1994; Kamio *et al.*, 1972a, b). The overall fatty acid pattern was consistent with the pattern found within members of the *Sporomusa–Pectinatus–Selenomonas* phyletic group (Strömpl *et al.*, 1999), but also allows differentiation of *C. quercicolum* from other members of this evolutionary group (Miyagawa *et al.*, 1979; Moore *et al.*, 1994; Shah *et al.*, 1983).

Cells for cytochrome determination were washed once with 100 mM phosphate buffer (pH 7.5) and disrupted by passing twice through a chilled French pressure cell at 18 000 p.s.i. (124 MPa). The membrane fraction was isolated from the homogenate by differential centrifugation (Wong & Jurtshuk, 1984) and the membranes were analysed spectrophotometrically by using a Varian DMS 100S dual-beam spectrophotometer as described previously (Wong & Maier, 1984). No reducible haem protein (i.e. cytochromes) could be detected in cell membranes. This is in contrast to the finding that the majority of species within the *Sporomusa–Pectinatus–Selenomonas* phyletic group contain both respiratory lipoquinones and cytochromes. It is interesting to note that the level of 'lipid F' found in *C. quercicolum* was about one-tenth of that

found in *Anaerovibrio* strains examined in parallel (Strömpl *et al.*, 1999). It has been established that cytochromes and respiratory lipoquinones, either in combination or individually, are components of respiratory electron transport in a wide range of aerobic and anaerobic organisms. The low levels of 'lipid F' and the absence of detectable amounts of haem proteins (i.e. cytochromes) in *C. quercicolum* suggests that this organism may differ in this respect from other members of the *Sporomusa–Pectinatus–Selenomonas* phyletic group.

Genetic analyses

Genomic DNA was prepared from fresh or freeze-dried cells by phenol/chloroform extraction (Wilson, 1987) or by a column-purification method (Qiagen Genomic Tips). Purified DNA was digested enzymically and the DNA base ratio was determined by HPLC (Tamaoka & Komagata, 1984) and calculated as described previously (Mesbah *et al.*, 1989). The G + C content of the chromosomal DNA was measured as 48.5 ± 0.3 mol % ($n=5$), which is lower than the 52–54 mol % (T_m) reported originally by Stankewich *et al.* (1971). This difference may be due to the different methods used. Either value is much higher than that observed in members of *Clostridium* cluster I, where G + C contents below 30 mol % are typical (Cato *et al.*, 1986). The genetic variation measured by the G + C base content is indicative of evolutionary divergence and changes in the nucleotide sequence of the genus (De Ley, 1967). A difference of 20 mol % in the DNA G + C base content between *C. quercicolum* and *Clostridium butyricum* confirms that the two are not related at the genus level.

The nearly complete 16S rRNA gene was amplified from genomic DNA by PCR (Mullis & Faloona, 1987) and the sequence was determined as described previously (Karlson *et al.*, 1993). The sequence of the 16S rDNA gene obtained in this study was identical to the sequence (accession number M59110) determined previously by reverse transcriptase sequencing of the 16S rRNA and deposited in databases (see Maidak *et al.*, 1999). Both sequences share the common feature that a stretch of nucleotides in helix 6 (Neefs *et al.*, 1991) could not be resolved, which, in our experience, indicates the existence of multiple rRNA gene operons with heterogeneities in the primary sequence structures of the individual operons (Rainey *et al.*, 1996).

Two spore-forming, Gram-negative genera in the *Sporomusa–Pectinatus–Selenomonas* phyletic group have been described to date that possess approximately 91.5% 16S rRNA gene sequence similarity to *C. quercicolum*. *Acetonea longum*, an acetogen from the termite gut (Kane & Breznak, 1991), has cells with a much greater length-to-width ratio, its motility is only observed on agar-coated slides and it is catalase-positive. It produces mainly acetate from H_2/CO_2 , but a mixture of end products including acetate, butyrate, propionate, succinate, propanol and H_2 from sugars

and organic acids, while *C. querciculum* ferments fructose to acetate, propionate, propanol and hydrogen (Cato *et al.*, 1986). The species of the genus *Sporomusa* (Breznak *et al.*, 1988; Dehning *et al.*, 1989; Hermann *et al.*, 1987; Kuhner *et al.*, 1997; Möller *et al.*, 1984; Ollivier *et al.*, 1985; Stackebrandt *et al.*, 1997) possess a different morphology, i.e. curved rods and a tuft of flagella on the concave side of the cell, while *C. querciculum* possesses only one to three single, lateral flagella (Fig. 1b, f). Species of *Sporomusa* do not form molecular hydrogen, they contain *b*-type cytochromes, most are catalase-positive, form acetate as the major fermentation product from a range of organic acids, betaine, methoxylated aromatic compounds and a few sugars, have a lower genomic DNA G+C content and are able, unlike *C. querciculum*, to grow chemoaerobically on H₂/CO₂. Considering these differences, we suggest the assignment of *C. querciculum* to a new genus as *Dendrosporobacter quercicolus* gen. nov., comb. nov.

Description of *Dendrosporobacter* gen. nov.

Dendrosporobacter (Den.dro.spo.ro.bac'ter. Gr. n. *dendro* tree; Gr. n. *spora* seed, spore; M.L. n. *bacter* equivalent of Gr. neut. n. *bakterion* rod, staff; M.L. masc. n. *Dendrosporobacter* a spore-bearing rod from a tree).

Cells are straight, motile rods with a Gram-negative cell wall. Endospores are formed. 'Lipid F', with octa- and nonaprenologues (ratio approx. 1:1) as the predominant isoprenologues, is present, but no cytochromes. Mesophilic. Predominant polar lipids are phosphatidyl ethanolamine, phosphatidyl serine and an unidentified glycolipid, together with a number of minor phospholipids and unidentified components. 3-Hydroxy fatty acids (3-OH 11:0, 3-OH 12:0 and 3-OH 13:0) are present in the fatty acids, approximately half of which are not esterified. The predominant non-hydroxylated fatty acids are 11:0, 15:1, 15:0 and 17:1. Obligately anaerobic. Catalase- and oxidase-negative. Chemo-organotroph. Major products in PY-fructose broth are acetate, propionate, propanol and H₂. The G+C content of the DNA is 48.5 mol% (HPLC) or 52–54 mol% (*T_m*). The type species is *Dendrosporobacter quercicolus* comb. nov.

Description of *Dendrosporobacter quercicolus* (Stankewich, Cosenza and Shigo 1971) comb. nov.

The description of *Dendrosporobacter quercicolus* comb. nov. is identical to that published for *Clostridium querciculum* (Stankewich *et al.*, 1971; Cato *et al.*, 1986) with the additions made in this publication. The type strain is DSM 1736^T.

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