***Clostridium uliginosum* sp. nov., a novel acid-tolerant, anaerobic bacterium with connecting filaments**

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An anaerobic, acid-tolerant bacterium, CK55^T, was isolated from an acidic forest bog. Cells of CK55^T stained Gram-negative but did not have an outer membrane. Cells were spore-forming, motile rods with peritrichous flagella, formed chains or aggregates and were linked by connecting filaments that were composed of a core and outer sheath. Cellobiose, glucose, xylose, mannose, mannitol, sucrose and peptone supported growth. Arabinose, lactose, raffinose, H₂/CO₂, CO/CO₂, vanillate, Casamino acids and various purines and pyrimidines did not support growth. Growth on carbohydrates yielded acetate, butyrate, lactate, formate and H₂ as end-products. Growth was observed at pH 4.0–9.0, with an optimum at pH 6.5, and at 10–30 °C, with an optimum at 20–25 °C. At 20 °C, doubling times were 4 and 6 h at pH 6.5 and 4.0, respectively. Hydrogenase activity in cell-free extracts was 12 U (mg protein)^{−1}. CK55^T did not: (i) contain detectable levels of CO, formate, lactate dehydrogenases or cytochromes; (ii) carry out dissimilatory reduction of nitrate or sulfate; or (iii) produce methane. Thus, CK55^T was characterized as a non-acetogenic, fermentative chemo-organotroph. The G+C content of CK55^T was 28.0 mol%. CK55^T was phylogenetically most closely related to *Clostridium botulinum* (types B, E and F), *Clostridium acetobutylicum* and other saccharolytic clostridia; the 16S rRNA gene sequence similarity values to the nearest relatives of CK55^T were approximately 97%. Based on morphological, physiological and phylogenetic properties of CK55^T, it is proposed that CK55^T be termed *Clostridium uliginosum* sp. nov. (= DSM 12992^T = ATCC BAA-53^T).

Keywords: acidic forest soil, acid tolerance, *Clostridium uliginosum*, connecting filaments

INTRODUCTION

Certain forest ecosystems are subjected to natural soil acidification and the effects of decades of acid deposition. Although there is some general information about soil micro-organisms and associated microbial processes in acidic soils, more detailed information on specific microbial processes and the identity of associated acid-tolerant microbes is less available (Robson & Abbott, 1989; Myrold & Nason, 1992).

The occurrence of anoxic microsites and anaerobic processes in soils and litter is well documented (Smith, 1980; Tiedje *et al.*, 1984; Sexton *et al.*, 1985; van der Lee *et al.*, 1999; Küsel & Drake, 1994, 1995; Wagner *et al.*, 1996; Küsel *et al.*, 1999). Under anoxic conditions, the production of CO₂ by acidic forest soil is spontaneous and is approximately one-third of that under oxic conditions (Matthies *et al.*, 1997). Although acidic soils exhibit anaerobic microbial potentials, including methanogenesis (Williams & Crawford, 1984; Goodwin & Zeikus, 1987; Crill *et al.*, 1988), denitrification (Shirey & Sexton, 1989; Blösl & Conrad, 1992; Nielsen *et al.*, 1994) and sulfate reduction (Wieder *et al.*, 1990), only a few acid-tolerant anaerobic micro-organisms have been characterized from such habitats. Acid-tolerant nitrate-reducing bacteria,

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a methanogen and saccharolytic clostridia have been isolated from acidic mine and forest soils, a peat bog and acidic lowland and forest bogs, respectively (Shirey & Sexstone, 1989; Blösl & Conrad, 1992; Williams & Crawford, 1985; Hammann & Ottow, 1976; Kuhner *et al.*, 2000).

In a recent study, acid-tolerant anaerobic bacteria were isolated from acidic forest sites and one of the novel isolates, *Clostridium akagii*, possessed a novel morphological structure termed a connecting filament (Kuhner *et al.*, 2000). Cells of *C. akagii* were often linked by these connecting filaments, which consisted of a core and surrounding sheath (Kuhner *et al.*, 2000). In the present study, a novel acid-tolerant, strictly anaerobic bacterium that also possesses connecting filaments was isolated from an acidic forest bog and characterized.

METHODS

Source of the organism. Bog samples were obtained from a forest site (Waldstein) in the Fichtelgebirge (spruce mountains) in east-central Germany; this site has been described previously (Matschonat & Matzner, 1996). Samples were collected from a bog area with peat accumulation. Characteristics of the peat bog were: pH 3.2; soil dry weight, 15%; organic carbon, 471 g kg⁻¹; carbon-to-nitrogen ratio, 17.8. Bog samples were collected in sterile glass containers and utilized immediately after transportation to the laboratory.

Isolation protocol. Bog samples were brought into a Mecaplex O₂-free chamber (100% N₂ gas phase; room temperature) and added to a reduced carbonate-buffered undefined medium (3 g wet weight bog sample per 50 ml medium). Enrichments were incubated at 15 °C and stable enrichments were obtained by repeated transfer in the same medium. After several transfers, enrichment cultures were streaked onto medium solidified with 1.5% agar. CK55^T was derived from an isolated colony and was transferred to liquid media and assayed for growth and substrate utilization.

Media and cultivation conditions. Anoxic media were prepared using modified techniques of Hungate (1969). For enrichments, a carbonate-buffered undefined medium was used that contained yeast extract, vitamins, trace metals, reducing agents (sodium sulfide and cysteine hydrochloride) and resazurin (redox indicator) (Daniel *et al.*, 1990). The pH was 6.9 and the medium was dispensed under 100% CO₂ into 120 ml serum bottles (50 ml medium per vial). The serum bottles were then sealed with butyl rubber stoppers and aluminium crimp seals and autoclaved. Anoxic tryptic soy broth (TSB) [Bacto tryptic soy broth without dextrose (Difco) at 5.5 g l⁻¹] was used as the standard cultivation medium for cultures of CK55^T. TSB was made anoxic by boiling and cooling under 100% argon. The pH was adjusted with H₃PO₄ or NaOH to the indicated pH and the medium was dispensed into 1 l infusion flasks (500 ml medium per flask) or 27 ml culture tubes (7 ml medium per tube) under 100% argon. The flasks or tubes were then sealed with butyl rubber stoppers and screw caps or aluminium crimp seals, respectively, and autoclaved. Aqueous stock solutions of substrates were prepared anoxically under argon, filter-sterilized and added by syringe injection using O₂-free

techniques. Unless otherwise indicated, CK55^T was incubated at 20 °C.

Alternative electron acceptors. The utilization of nitrate or sulfate was determined using TSB (30 g l⁻¹) supplemented with 10 mM glucose and 5 mM NaNO₃ or 10 mM Na₂SO₄, respectively. The capacity to grow aerobically was evaluated on Nutrient Agar (Difco) supplemented with 10 mM glucose; the agar plates were incubated under air.

Electron microscopy. Cells were cultivated in liquid media or on solidified media [1% Gelrite (Carl Roth)] supplemented with 10 mM glucose. Cells were negatively stained with uranyl acetate (Valentine *et al.*, 1968). For thin sections, cells were fixed in glutaraldehyde/OsO₄ and prepared by a standard protocol (Traub *et al.*, 1976). Thin sections were stained with 2% (w/v) aqueous uranyl acetate and lead citrate (Reynolds, 1963). Specimens were observed with a Zeiss CEM 902A.

Preparation of cell-free extract and enzyme assays. Cell-free extracts were prepared under anoxic conditions as described previously (Lundie & Drake, 1984; Kuhner *et al.*, 2000). Enzyme activities were assayed spectrophotometrically (Drake, 1982) at 20 °C. The Tris/HCl (50 mM, pH 7.5) enzyme assay buffer contained 0.5 mM benzyl viologen and was supplemented with H₂ (20% in N₂) for hydrogenase, CO (20% in N₂) for CO dehydrogenase, sodium formate (4 mM) for formate dehydrogenase or sodium lactate (4 mM) for lactate dehydrogenase. Hydrogenase activity was also determined in the same assay buffer containing benzyl viologen that had been reduced with sodium dithionite; the gas phase was 100% N₂ and H₂ formation was verified by GC.

Membrane preparation and redox difference spectra. Membranes and cytoplasmic fractions were prepared under oxic conditions (Fröstl *et al.*, 1996). Washed membranes and cytoplasmic fractions were reduced with sodium dithionite and reduced-minus-oxidized spectra were obtained at room temperature with an Uvikon 930 (Kontron Instruments) double-beam recording spectrophotometer (Kuhner *et al.*, 1997).

G + C content. Cells were treated with penicillin G (250 µg ml⁻¹) 3 h before harvesting. DNA extraction was performed by a method modified after Marmur (1961) and included treatments with lysozyme/proteinase K and RNase/proteinase K. The G + C content was determined by HPLC (Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984).

Analysis of 16S rRNA gene sequence. A total of 1437 bases of the 16S rRNA gene of CK55^T was sequenced by direct sequencing of the PCR-amplified 16S rRNA gene. DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR products were performed according to published protocols (Marmur, 1961; Sambrook *et al.*, 1989; Dorsch & Stackebrandt, 1992). Cells were lysed using lysozyme. Purified PCR products were sequenced using the *fmoI* DNA Cycle Sequencing kit (Promega) and ³²P-labelled primers as described by the manufacturer. Sequencing reactions were electrophoresed on 6% and 8% acrylamide wedge-shaped gels using a Sequi-Gen GT Sequencing Cell (Bio-Rad). Autoradiograms were obtained by exposing X-ray films (Kodak BioMax MR). Alignment of the sequence data was performed using the tools of the ARB software package for automated alignment (Strunk *et al.*, 1998). The distance-matrix method was used for calculation of sequence similarities; distance-matrix, maximum-parsimony and maximum-likelihood methods

were applied for tree reconstruction as implemented in the ARB software package. Different data sets were analysed, varying with respect to the selection of outgroup reference sequences.

Additional analytical methods. Growth was measured as OD₆₆₀; the optical path width (inner diameter of culture tubes) was 1.6 cm. Uninoculated medium served as a reference. Protein in cell-free extracts was determined by the method of Bradford (1976). The amounts of substrates and products present in culture fluids and gas phases were determined by HPLC and GC (Daniel *et al.*, 1990; Gößner *et al.*, 1994; Küsel & Drake, 1995; Matthies *et al.*, 1993). Soil pH was determined in 1:2.5 suspensions of soil in 0.02 M CaCl₂. Nitrate was measured colorimetrically (Cataldo *et al.*, 1975). Results and values are representative of duplicate experiments.

RESULTS

Cell morphology

Cultures of CK55^T contained single rods and formed multicellular chains or aggregates. Cells were 8 × 1 µm and had two to four peritrichous flagella (Fig. 1a). CK55^T stained Gram-negative; however, the organism had no outer membrane (Fig. 1b). CK55^T formed terminal spores (Fig. 1c). Free spores were rarely observed in cultures.

Connecting filament

Cell chains consisted of up to five to seven cells that were often tethered to one another by a connecting filament (Fig. 2a). The connecting filament consisted

of a core and a surrounding sheath (Fig. 2b–d). The sheath was not always present, indicating that it could be dissociated from the core. The core had a tubular structure and appeared to have a small inner channel (Fig. 2e).

Effects of temperature and pH on growth

In glucose-supplemented TSB (pH 6.9), the temperature range for growth of CK55^T was 10–30 °C. The temperature optimum was 20–25 °C. Growth occurred when cells were pasteurized at 72 °C for 15 s; growth did not occur when cells were heated at 80 °C for 15 min.

CK55^T exhibited a broad pH range; growth occurred at pH 4.0–9.0. Growth did not occur at pH 3.5 or 10.0. Growth at pH 9.0 occurred after a lag phase of 2 d. The doubling times were 6, 4 and 7 h at pH 4.0, 6.5 and 8.0, respectively. When CK55^T was cultivated in media having an initial pH of 4.0 or 4.5, the pH of the medium did not change significantly during growth. In contrast, the pH of the medium decreased by up to 4 units during growth when CK55^T was cultivated in media having an initial pH between 5.0 and 9.0; the final pH in such cases was 4.6–5.0.

Substrate range and fermentation products

In TSB (pH 6.0), glucose, mannose, xylose, mannitol, cellobiose, sucrose and peptone supported growth of CK55^T. In TSB (pH 6.0) supplemented with 10 mM

Fig. 1. Transmission electron micrographs of CK55^T. (a) Negatively stained preparation (F, flagella; C, core of connecting filament; S, septum); bar, 0.5 µm. (b) Thin section (CW, cell wall; CM, cytoplasmic membrane); bar, 0.005 µm. (c) Thin section, showing formation of a terminal spore (SP, spore); bar, 0.2 µm.

Fig. 2. Transmission electron micrographs of connecting filaments of CK55^T cells. Abbreviations: C, core; CF, connecting filament; Sh, sheath; T, tube. (a–c) Negatively stained preparations; bars, 2, 0.5 and 0.2 μ m, respectively. (d) Negatively stained preparation. The large arrow points to the central structure of the core; bar, 0.01 μ m. (e) Negatively stained preparation. The large arrow points to a channel opening of a broken tube; bar, 0.005 μ m.

Table 1. Product profiles of CK55^T when cultivated on various substrates

Substrate	Substrate consumed (mM)	Maximum OD ₆₆₀	Products formed (mM)*					H recovery (%)†
			Acetate	Butyrate	Formate	Lactate	H ₂	
Glucose	6.2	0.94	2.5	3.4	0.0	0.4	22.3	93
Mannose	7.4	1.05	3.6	4.7	0.6	0.4	20.3	96
Cellobiose	2.9	1.02	2.9	3.9	0.0	0.3	16.9	100
Xylose	8.4	1.07	3.8	6.0	1.2	0.0	21.8	117

* CO₂ was not determined.

† Reducing equivalents.

mannose, concomitant formation of H₂, butyrate and acetate was observed throughout exponential growth (which lasted approx. 20 h); formate and lactate were trace products (Table 1; data not shown). Although not analysed, CO₂ was very likely produced as well. Similar product profiles were obtained with glucose, cellobiose and xylose (Table 1).

Arabinose, maltose, lactose, glycerol, raffinose, Casamino acids, adenine, cytosine, uracil, nicotinic acid, xanthine, H₂/CO₂, CO/CO₂ and vanillate did not support growth of CK55^T. The organism did not carry out dissimilatory reduction of nitrate or sulfate, produce methane or grow aerobically on glucose-supplemented nutrient agar.

Fig. 3. Phylogenetic tree of the 16S rRNA genes of CK55^T and strains of related *Clostridium* species. *Caloramator fervidus*, *Ruminococcus albus* and *Bacillus licheniformis* are shown as outgroups. The tree topology was evaluated and corrected by applying maximum-parsimony, maximum-likelihood and distance-matrix analyses using the tools of the ARB software package. The multifurcation indicates that a common significant relative branching order could not be found. Accession numbers are given in parentheses. Bar, 10 nt substitutions per 100 sequence positions.

Enzyme activities and G + C content

With cell-free extracts of glucose-grown cells of CK55^T, hydrogenase activity was only detected in the direction of H₂ formation in the pre-reduced benzyl viologen-containing assay. Based on the oxidation rate of benzyl viologen, hydrogenase activity was approximately 11.9 µmol min⁻¹ (mg protein)⁻¹. CO, formate and lactate dehydrogenases were not detected. No cytochromes were detected in the particulate or soluble fractions of glucose-grown cells of CK55^T.

The G + C content of the DNA of CK55^T was 28.0 ± 0.3 mol % (*n* = 3).

Phylogenetic analysis

Phylogenetic analysis of the 16S rRNA gene sequence of CK55^T indicated that the organism belonged to cluster I of the genus *Clostridium*. The organism was most closely related to various saccharolytic clostridia (Fig. 3). The 16S rRNA gene sequence similarity values to *Clostridium botulinum* ATCC 9564 (type E), *C. botulinum* ATCC 23387 (type F) and *C. botulinum* ATCC 25765 (type B) were respectively 97.3, 97.3 and 97.2%. The 16S rRNA gene sequence similarity values to *Clostridium acetobutylicum* NCP 262, *Clostridium puniceum* DSM 2619^T, ‘*Clostridium favosporum*’ DSM 5907 and ‘*Clostridium corinoforum*’ DSM 5906 were 97.1, 96.8, 96.7 and 96.7%, respectively. The 16S rRNA gene sequence similarity value to *C. akagii*

DSM 12554^T, the only other organism known to form connecting filaments, was 92.4%.

DISCUSSION

Phylogenetic analysis of the 16S rRNA gene sequence indicated that CK55^T grouped within cluster I of the genus *Clostridium* (Collins *et al.*, 1994). The difference between the 16S rRNA gene sequence of CK55^T and that of its closest phylogenetic relatives was approximately 3%, indicating that CK55^T constituted a novel clostridial species (Stackebrandt, 1997). CK55^T was a mesophilic anaerobe and fermented carbohydrates to acetate, butyrate and H₂, as well as small amounts of other products, including formate and lactate (Table 1). Thus, CK55^T was physiologically similar to other saccharolytic cluster I clostridial species (Cato *et al.*, 1986).

In contrast to *C. botulinum* (types E and F), CK55^T could be cultivated on cellobiose, mannitol or xylose (Cato *et al.*, 1986). CK55^T grew well at pH 4.0 and displayed a broad pH range of growth. The ability of non-proteolytic strains of *C. botulinum* (types E and F) to grow at low pH is unknown to us. Growth of proteolytic strains of *C. botulinum* (types A and B) at pH 4 only occurs after protracted incubation times (Raattjes & Smelt, 1979). CK55^T was isolated from a water-saturated, acidic forest bog, a habitat from which *C. botulinum* has not been isolated. In addition,

CK55^T and *C. botulinum* have different morphologies. Spores of CK55^T were formed terminally, whereas *C. botulinum* has subterminal spores (Fig. 2c; Cato *et al.*, 1986; Smith, 1992). Cells of CK55^T were often tethered by connecting filaments (Fig. 1), a morphological structure that has not been observed in *C. botulinum*. Collectively, the morphological, physiological and phylogenetic properties of CK55^T indicate that the organism is a novel species within cluster I of the genus *Clostridium* and the name *Clostridium uliginosum* sp. nov. is proposed for CK55^T.

Acid-tolerance among the clostridia has not been evaluated extensively and there are only a few clostridia that are known to grow at low pH, i.e. pH 4.0–4.5 (Raattjes & Smelt, 1979; Gibson, 1965; Schwartz & Keller, 1982). With respect to clostridia isolated from acidic soils, *C. acetobutylicum*, *Clostridium butyricum*, *Clostridium saccharobutyricum*, *Clostridium acidisoli* and *C. akagii* are able to grow at pH 4 or lower (Hammann & Ottow, 1976; Kuhner *et al.*, 2000). Other acid-tolerant bacteria within clostridial cluster I are the anaerobic bacteria *Sarcina maxima* and *Sarcina ventriculi*, which can grow at a pH as low as 2 (Canale-Parola, 1986).

Connecting filaments were first described for *C. akagii* (Kuhner *et al.*, 2000). With CK55^T, a more detailed, tubular structure of the connecting filament core has been observed. It appears that a tubular channel occurs in the inner core of the filament. Whether or not this channel represents a cytoplasmic connection between cells is not yet clear. That connecting filaments have been observed with both *C. akagii* and CK55^T demonstrates that this cellular structure is not unique to one species. However, the extent of its distribution, its chemical composition and its ecophysiological role(s) have yet to be determined.

Description of *Clostridium uliginosum* sp. nov.

Clostridium uliginosum (u.li.gi.no'sum. L. n. *uligo* -inis moisture of the soil, swamp; L. neut. adj. *uliginosum* swampy).

Cells are rods, 8 × 1 µm, motile, have two to four peritrichous flagella per cell, are spore-forming, lack an outer membrane and stain Gram-negative. Cultures contain single cells and cell chains; cells are linked by a connecting filament that is composed of a core and an outer sheath. Anaerobe with a chemo-organotrophic, fermentative metabolism. Grows on glucose, cellobiose, sucrose, xylose, mannose, mannitol and peptone. Glucose is fermented to acetate, butyrate, lactate, H₂ and CO₂; with other sugars, formate is also formed. Does not grow on lactose, maltose, glycerol, arabinose, raffinose, Casamino acids, H₂/CO₂, CO/CO₂, vanillate or various purines or pyrimidines. Does not carry out dissimilatory reduction of nitrate or sulfate. Grows at 10–30 °C, with an optimum at 20–25 °C. Grows at pH 4.0–9.0, with an optimum at pH 6.5. At 20 °C, doubling times are 6, 4 and 7 h at pH

4.0, 6.5 and 8.0, respectively. Cells do not contain cytochromes. G+C content is 28.0 mol%. Phylogenetically most closely related to *Clostridium botulinum* (types B, E and F), *Clostridium acetobutylicum* and other saccharolytic clostridia. The type strain, CK55^T (= DSM 12992^T = ATCC BAA-53^T), was isolated from an acidic forest bog.

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