***Clostridium akagii* sp. nov. and *Clostridium acidisoli* sp. nov.: acid-tolerant, N₂-fixing clostridia isolated from acidic forest soil and litter**

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Two anaerobic acid-tolerant bacteria, CK58^T and CK74^T, were isolated from acidic beech litter and acidic peat-bog soil, respectively. Both bacteria were spore-forming, motile rods with peritrichous flagella. The capacity to sporulate decreased with prolonged cultivation. Cells of CK58^T formed chains or aggregates and were linked by a connecting filament that consisted of a core and a surrounding sheath. Cellobiose, glucose, xylose, arabinose, maltose, mannose and salicin supported growth of CK58^T. These substrates, as well as mannitol, lactose, sucrose, glycerol, melezitose, raffinose and rhamnose, supported growth of CK74^T. Sorbitol, trehalose, H₂/CO₂, CO/CO₂, vanillate, Casamino acids, peptone, and various purines and pyrimidines did not support the growth of either organism. Growth of CK58^T and CK74^T on glucose yielded butyrate, lactate, acetate, formate, H₂ and CO₂ as end products. Growth of CK58^T and CK74^T was observed at pH 3.7–7.1 and 3.6–6.9, respectively. CK58^T and CK74^T grew in nitrogen-free medium at pH 3.7 under an N₂ atmosphere and reduced acetylene at rates approximating 1 nmol min⁻¹ (mg protein)⁻¹. CK58^T and CK74^T did not contain carbon monoxide dehydrogenase or cytochromes, produce methane, or dissimilate nitrate or sulfate. Thus, CK58^T and CK74^T were characterized as nonacetogenic, N₂-fixing, fermentative chemo-organotrophs. The G+C contents of CK58^T and CK74^T were 31.4 and 30.7 mol%, respectively. CK58^T and CK74^T were phylogenetically most closely related to *Clostridium pasteurianum*. The 16S rRNA gene sequence similarity values of CK58^T and CK74^T to *C. pasteurianum* and each other did not exceed 96.5%, and it is proposed that strains CK58^T and CK74^T be named *Clostridium akagii* CK58^T (DSM 12554^T) and *Clostridium acidisoli* CK74^T (DSM 12555^T), respectively. These results suggest that previously uncharacterized clostridial species reside and might fix N₂ in the anoxic microzones of acidic forest soil and litter.

Keywords: acidic forest soil, acid tolerance, *Clostridium acidisoli*, *Clostridium akagii*, nitrogen fixation

INTRODUCTION

Increasing concern about the effects of acid deposition on forest ecosystems has stimulated research on the short-term effects of soil acidification on soil micro-

organisms and associated microbial processes (Robson & Abbott, 1989; Myrold & Nason, 1992). However, the microbiology of naturally acidic soils or soils subjected to decades of acidification has been less well characterized (Myrold & Nason, 1992).

Anoxic microsites exist within soils (Smith, 1980; Sextone *et al.*, 1985) and needle litter (van der Lee *et al.*, 1999), and the spontaneous capacity of acidic soils to produce CO₂ under anoxic conditions is approxi-

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mately one-third of that under oxic conditions (Matthies *et al.*, 1997). A number of anaerobic processes including methanogenesis (Williams & Crawford, 1984; Goodwin & Zeikus, 1987; Crill *et al.*, 1988), denitrification (Shirey & Sexstone, 1989; Blösl & Conrad, 1992; Nielsen *et al.*, 1994) and sulfate reduction (Wieder *et al.*, 1990) have been demonstrated to occur in acidic soils. However, only a few organisms capable of anaerobic growth at low pH have been characterized from such environments. An acid-tolerant methanogen has been isolated from a Minnesota bog (Williams & Crawford, 1985), several acid-tolerant, nitrate-reducing bacteria have been isolated from acidic mine and forest soils (Shirey & Sexstone, 1989; Blösl & Conrad, 1992) and a couple of acid-tolerant, saccharolytic clostridia have been isolated from an acidic gley soil (Hammann & Ottow, 1976). In the present study, two new N_2 -fixing, acid-tolerant clostridia were isolated and characterized during efforts to determine the general occurrence and acid tolerance of obligately anaerobic bacteria in soil and litter from a forest subject to long-term acidic conditions.

METHODS

Litter and soil. Litter and soil samples were obtained from three forest sites in the Fichtelgebirge (spruce mountains) in east-central Germany. Upper soil horizons at these sites exhibited an approximate pH of 3. Beech litter and Ah horizon soil (the deeper organic layer in which fine organic matter dominates) were collected at the Schneeberg-Schacht site which is populated primarily by beech (*Fagus sylvatica*) (Matschonat & Matzner, 1996). At the Waldstein site, peat-bog soil was collected from a bog area with peat accumulation and Oh horizon soil (the mineral layer which contains up to 15% humus) was collected from the Waldstein/Coulissenhieb site which is populated primarily by Norway spruce (*Picea abies*). These sites have been described previously by Matschonat & Matzner (1996). Characteristics of the peat-bog soil were: soil dry weight, 15%; organic carbon, 471 g kg⁻¹; carbon to nitrogen ratio, 17.8. Litter and soils were collected in sterile glass containers and utilized immediately after transportation to the laboratory.

Enrichment cultures and isolation protocol. Litter and soil samples were brought into a Mecaplex O_2 -free chamber (100% N_2 gas phase; room temperature) and added to anoxic media [3 g (wet weight) soil or litter per 50 ml medium]. To enrich for spore formers, soils or litter were pasteurized (7 min at 95 °C) in sterile anoxic buffer [20 g soil or litter (wet weight) per 80 ml buffer] prior to being added to media. The anoxic buffer contained (mg l⁻¹): $NaHCO_3$, 7500; KH_2PO_4 , 500; $MgCl_2 \cdot 6H_2O$, 50; NaCl, 400; $CaCl_2 \cdot 2H_2O$, 10; resazurin, 1. The buffer components were boiled and cooled under 100% CO_2 , then autoclaved. Enrichments were incubated at 15 °C, and stable enrichments were obtained by repeated transfer in the indicated medium. After several transfers, enrichment cultures were streaked onto media solidified with 1.5% agar. Isolated colonies were transferred to liquid medium and assayed for growth and substrate utilization.

Media and cultivation conditions. Anoxic reduced media

were prepared using modified Hungate techniques (Hungate, 1969). The carbonate-buffered undefined medium contained yeast extract, vitamins, trace metals, reducer (sodium sulfide and cysteine hydrochloride) and resazurin (redox indicator) (Daniel *et al.*, 1990). The pH was adjusted with H_2SO_4 to pH 4 or 5 and the medium was dispensed under 100% CO_2 into 120 ml serum bottles (50 ml medium per vial) or 27 ml culture tubes (7 ml medium per tube). The serum bottles or tubes were then sealed with butyl rubber stoppers and aluminium crimp seals, and autoclaved. The defined medium was the undefined medium without yeast extract. The phosphate-buffered defined medium was the defined medium with KH_2PO_4 (14 g l⁻¹) instead of bicarbonate; the medium was prepared under 100% argon. Aqueous stock solutions of substrates were prepared anoxically under argon, filter sterilized and added by syringe injection using O_2 -free techniques. API identification strips (bioMérieux) were incubated in O_2 -free jars.

Maintained cultures of CK58^T and CK74^T. CK58^T and CK74^T were isolated from enrichments derived from pasteurized (see above) litter and bog soil, respectively, and were maintained in the undefined medium (see above) adjusted to pH 6.8 or anoxic tryptic soy broth (TSB) medium. TSB contained 5.5 g Bacto tryptic soy broth without dextrose (Difco) per litre. The medium was prepared by boiling and cooling under 100% argon. The pH was adjusted with H_3PO_4 or NaOH to the indicated pH, and the medium was dispensed under 100% argon. Unless otherwise indicated, CK58^T and CK74^T were incubated at 20 and 30 °C, respectively.

Alternative electron acceptors. The utilization of nitrate was determined using TSB medium (at full strength for CK58^T or at a 1:10 dilution for CK74^T) supplemented with 10 mM glucose and 5 mM $NaNO_3$. The utilization of sulfate was determined in medium containing lactate and sulfate (Postgate, 1984). The capacity to grow aerobically was evaluated on nutrient agar (Difco) supplemented with 10 mM glucose; the agar plates were incubated under air.

Fixation of N_2 and reduction of acetylene. The fixation of N_2 was determined using a nitrogen-free medium designed to assess N_2 -fixing micro-organisms (combined carbon medium; Rennie, 1981). The medium was modified by replacing yeast extract with a trace-element solution (Alef, 1991) (1 ml l⁻¹), omitting sucrose and adding glucose to 10 mM. The medium was prepared anoxically with a 100% argon gas phase, adjusted to pH 3.7 and dispensed under 100% argon. Cells grown with N_2 as the sole source of nitrogen were tested for their ability to reduce acetylene by adjusting the gas phase of culture tubes to 10% acetylene (Hardy *et al.*, 1972). Gas samples were withdrawn periodically with sterile syringes and the production of ethylene was determined by GC (Limmer & Drake, 1996).

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- OsO_4 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 transmission electron microscope.

Preparation of cell-free extract and enzyme assays. Two litres of glucose-grown cultures were dispensed into centrifuge bottles in a Mecaplex O_2 -free chamber (100% N_2 gas

phase; room temperature); the bottles were sealed and centrifuged (14 300 g, 30 min, 4 °C). The cell pellet was washed with anoxic sodium phosphate buffer (50 mM, pH 7) and recentrifuged. The washed cell pellet was resuspended in 1.5 vols anoxic lysozyme buffer (Lundie & Drake, 1984), incubated for 1 h at room temperature and centrifuged at 16 000 g (20 min, 4 °C). The supernatant fluid was transferred to a serum vial (100% N₂ gas phase) and assayed immediately. Enzyme activities were assayed spectrophotometrically (Drake, 1982a) at 20 °C for CK58^T and at 30 °C for CK74^T. 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 carbon monoxide dehydrogenase or 4 mM formate for formate dehydrogenase.

Membrane preparation and redox-difference spectra. Membranes and cytoplasmic fractions were prepared under oxic conditions (Frösl 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. DNA was extracted and purified by standard methods (Cashion et al., 1977). The G + C content was determined by HPLC (Mesbah et al., 1989).

Analysis of 16S rRNA gene sequence. A total of 1492 and 1498 bases of the 16S rRNA genes of CK58^T and CK74^T, respectively, were sequenced by direct sequencing of the PCR-amplified 16S rRNA genes. 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 fmol DNA cycle sequencing kit (Promega) and ³³P-labelled primers as described by the manufacturer. Sequencing reactions were electrophoresed on 6% and 8% acrylamide wedge-shaped gels using the Sequi-Gen GT sequencing cell (Bio-Rad). Autoradiograms were obtained by exposing X-ray films (Kodak BioMax MR). Alignments of the sequence data were performed manually, and determination of similarity values was done with the ae2 editor (Maidak et al., 1996).

Additional analytical methods. Growth was measured as OD₆₆₀; the optical path length was (inner diameter of culture tubes) 1.6 cm. Uninoculated medium served as a reference. Protein in cell-free extracts was determined by the Bradford method (Bradford, 1976). The protein content of cells in culture was estimated by using the experimentally determined conversion factors of 375 (CK74^T) and 340 (CK58^T) mg (cell dry weight) l⁻¹ at an OD₆₆₀ of 1.0; protein was assumed to constitute 50% of the cell dry weight (Madigan et al., 1997). The amounts of substrates and products present in culture fluids and gas phases were determined by HPLC and GC (Daniel et al., 1990; Göbner 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.

Culture numbers. Cultures of CK58^T and CK74^T have been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as DSM 12554 and DSM 12555, respectively.

RESULTS

Enrichment and isolation

Twenty-two bacterial isolates capable of anaerobic growth on glucose were obtained from enrichment cultures with the undefined, defined and phosphate-buffered media at pH 4 and 5. Fifteen isolates were facultative anaerobes and seven isolates were strict anaerobes. Using the API identification system (20E strips), several facultative organisms were identified as species of *Enterobacter* or *Serratia*. Of the seven anaerobes, two that were isolated from peat-bog soil (CK64 and CK74^T) and one that was isolated from beech litter (CK58^T) grew on the API 20A medium; however, only CK64 could be identified (*Actinomyces israelii*). CK58^T and CK74^T were identified by 16S rRNA gene sequencing (see below). The identities of the four anaerobes that did not grow on the API 20A medium have not been determined.

Phylogenetic analyses

The phylogenetic analyses of the 16S rRNA gene sequences of CK58^T and CK74^T indicated that both organisms belonged to cluster I of the genus *Clostridium*. Both organisms were most closely related to *Clostridium pasteurianum* (Fig. 1). The 16S rRNA gene sequence similarity values to *C. pasteurianum* and to each other were not greater than 96.5%, indicating

Fig. 1. Phylogenetic tree of the 16S rRNA genes of CK58, CK74 and strains of related *Clostridium* species. The tree was calculated by the distance matrix algorithm of DeSoete (1983), including the compensation for multiple substitutions that may occur at the same site (Jukes & Cantor, 1969). The percentage of 500 bootstrap resemblings that support branching points above 80% confidence is indicated. The scale bar corresponds to 5 nucleotide substitutions per 100 sequence positions. Numbers in parentheses indicate accession numbers. The tree was rooted with *Bacillus subtilis* as an outgroup (Maidak et al., 1997).

Fig. 2. Transmission electron micrographs of CK58^T. (a, b) Negatively stained preparations. The large arrow in (b) points to a separated sheath. Abbreviations: CF, connecting filament; C, core; Sh, sheath; S, septum. (c) Thin section. Abbreviations: WL1, wall layer 1; WL2, wall layer 2; CM, cytoplasmic membrane; S, septum. Bars: 0.5 µm (a), 0.1 µm (b), 1 µm (c).

that CK58^T and CK74^T belonged to new clostridial species.

Morphological and cytological characteristics

CK58^T and CK74^T were motile rods, and cells were 2–11 × 1 µm and 3–7 × 1 µm, respectively. Cells of CK58^T and CK74^T had 10–15 and 8–12 peritrichous flagella, respectively (Figs 2a and 3a). Upon isolation, both organisms formed spores. However, the capacity to sporulate decreased with prolonged cultivation, especially in the case of CK58^T. The spores of CK74^T were oval and terminal. CK58^T stained Gram-negative and CK74^T stained Gram-positive; however, neither organism had an outer membrane (Figs 2c and 3b).

Cultures of both organisms contained single rods and multicellular chains or aggregates. Chains of CK74^T consisted of up to 14 cells that were not separated after septum formation (Fig. 3c, d). Cells of CK58^T were often tethered to one another by a connecting filament (Fig. 2b). The connecting filament consisted of two

morphologically distinct entities, namely the core and the sheath; the sheath was often separated from the core during preparation of the cells for microscopy (Fig. 2b). The connecting filament facilitated the formation of cell chains as well as net-like cell aggregates (Fig. 2b).

On TSB agar at pH 5, CK58^T formed irregular colonies, generally 1.5–2 mm in diameter. Young colonies were white, opaque and shiny, with undulate margins. Older colonies became beige and umbonate, and were brown in the centre. CK74^T was similar, but colonies were mucoid and 2–3 mm in diameter.

Optimal growth conditions

In glucose-supplemented TSB medium (pH 4), the temperature ranges for CK58^T and CK74^T were 5–30 °C and 5–37 °C, respectively. The temperature optima of CK58^T and CK74^T were 20–25 °C and 25–30 °C, respectively. CK58^T exhibited a broad pH range of pH 3.7–7.1 with no distinct optimum between

Fig. 3. Transmission electron micrographs of CK74^T. (a) Negatively stained preparation. Abbreviations: F, flagella; C, capsule-like layer consisting of fibrillar material with little defined structure. (b) Thin section. Abbreviations: C, capsule-like layer; WL1, wall layer 1; WL2, wall layer 2; CM, cytoplasmic membrane; I, inclusion body. (c) Thin section showing septum formation at two division points (S1 and S2) of a filamentous cell. (d) Negatively stained preparation. Bars: 0.5 µm (a), 0.1 µm (b), 0.5 µm (c), 1 µm (d).

pH 4.2 and 6.8 (Fig. 4). The pH range of CK74^T was pH 3.6–6.9 with no distinct optimum between pH 3.6 and 6.6 (data not shown). In glucose-supplemented TSB medium (pH 4), the doubling times of CK58^T (25 °C) and CK74^T (30 °C) approximated 11 and 3.5 h, respectively.

Change in pH during growth

When CK58^T and CK74^T were cultivated in media having an initial pH of 3.7 and 3.6, respectively, the pH of the media decreased approximately 0.2 pH units during growth. In contrast, when CK58^T and CK74^T were cultivated in media having an initial pH of 6.8 and 6.9, respectively, the pH of the media decreased approximately 2.3 pH units during growth.

Fermentation products and substrate range

In TSB medium (pH 5.5), glucose was fermented to acetate, lactate, butyrate, H₂ and CO₂ by CK58^T and

CK74^T (Table 1); the same products were formed in glucose-supplemented TSB medium at pH 4.0 and 6.5 (data not shown). In the undefined medium at pH 6.8, formate was also produced (Table 1). Neither organism produced methane.

API analyses indicated that cellobiose, xylose, arabinose, maltose, mannose and salicin supported the growth of CK58^T; mannitol, lactose, sucrose, glycerol, melezitose, raffinose, rhamnose, sorbitol and trehalose did not support growth. With CK74^T, all of these substrates supported growth except sorbitol and trehalose. H₂, CO or vanillate were not consumed or growth supporting for CK58^T and CK74^T in TSB (prepared with 100% CO₂) and undefined medium, respectively. In TSB medium, Casamino acids, peptone, adenine, cytosine, uracil, nicotinic acid and xanthine did not support the growth of CK58^T and CK74^T. Both isolates hydrolysed aesculin but not gelatin. Neither organism utilized nitrate or sulfate

Fig. 4. Effect of pH on the growth of CK58^T in TSB medium supplemented with 10 mM glucose. Initial pH of medium: ▲, 2.6; △, 3.2 and 7.4; ●, 3.7; ○, 4.2; □, 4.6; ▼, 5.4; +, 6.8; ■, 7.1.

and neither organism grew aerobically on glucose-supplemented nutrient agar.

N₂ fixation at low pH

Cultures of CK58^T and CK74^T were maintainable in nitrogen-free medium at pH 3.7 under an N₂ atmosphere; no growth was observed in the nitrogen-free medium under an argon atmosphere (Fig. 5). Cells of CK58^T and CK74^T that were grown in medium with N₂ as the sole source of nitrogen reduced acetylene at rates approximating 0.6 and 0.7 nmol min⁻¹ (mg protein)⁻¹, respectively.

Enzyme activities

No cytochromes were detected in the particulate and soluble fractions of glucose-grown cells of CK58^T and CK74^T.

Cell-free extracts of glucose-grown cells of CK58^T and CK74^T had similar hydrogenase activities [3.6 and 2.6 µmol min⁻¹ (mg protein)⁻¹, respectively]. Carbon monoxide dehydrogenase was not detectable and formate dehydrogenase activities were either not detectable (CK74^T) or negligible [0.005 µmol min⁻¹ (mg protein)⁻¹; CK58^T]. Neither isolate contained catalase, oxidase, tryptophanase or urease activities.

G + C content

The G + C contents of the DNA of CK58^T and CK74^T were 31.4 ± 0.2 (*n* = 3) and 30.7 ± 0.1 mol % (*n* = 3), respectively.

DISCUSSION

Phylogenetic analyses of the 16S rRNA gene sequences indicated that CK58^T and CK74^T are new species within cluster I of the genus *Clostridium* (Collins *et al.*, 1994). Morphologically and physiologically, the isolates differed markedly from each other and from their closest phylogenetic relative, *C. pasteurianum*. CK74^T has a much broader range of utilizable substrates and a faster growth rate than CK58^T. Cells of CK74^T did not separate after septum formation; in contrast, connecting filaments were observed with CK58^T. Both organisms differed from *C. pasteurianum* in substrate range, the formation of lactate but not ethanol, growth and N₂ fixation at pH 3.7, and the lack of carbon monoxide dehydrogenase (Cato *et al.*, 1986). CK58^T and CK74^T are therefore proposed to be new bacterial species and be named *Clostridium akagii* and *Clostridium acidisoli*, respectively.

Nickel-containing carbon monoxide dehydrogenases oxidize CO to CO₂ and function in acetogenic bacteria as acetyl-CoA synthase (Drake, 1994; Wood & Ljungdahl, 1991). Thus, the occurrence of carbon monoxide dehydrogenase in the genus *Clostridium* has mostly been observed in acetogenic clostridia such as *Clostridium acetium*, the first acetogen to be isolated

Table 1. Glucose-dependent product profiles of CK58^T and CK74^T

ND, not determined.

Strain	Medium (pH)	Glucose consumed (mM)	Max. OD ₆₀₀	Products formed (mM)						Recovery (%)	
				Butyrate	Lactate	Acetate	Formate	CO ₂	H ₂	C	H*
CK58 ^T	TSB (5.5)	11.2	0.793	6.3	2.6	5.3	0	18.2	21.2	92	90
CK74 ^T	TSB (5.5)	10.4	0.986	5.3	3.0	4.7	0	17.2	19.1	91	87
CK58 ^T	Undefined (6.8)	10.4	0.462	6.1	2.1	3.9	3.2	ND	18.7	ND	88
CK74 ^T	Undefined (6.8)	9.4	0.498	3.8	4.7	3.8	5.4	ND	10.4	ND	86

* Reducing equivalents.

Fig. 5. Effect of different nitrogen sources on the growth of CK58^T (a) and CK74^T (b) in nitrogen-free medium (pH 3.7) supplemented with 10 mM glucose. Nitrogen sources: ●, ammonium chloride (7 mM); ○, 100% N₂ gas phase; ■, no N source. Inocula were derived from a culture that had been transferred three times in medium with N₂ as the sole source of nitrogen.

(Wieringa, 1936). Although the occurrence of carbon monoxide dehydrogenase in the nonacetogen *C. pasteurianum* is paradoxical (Diekert *et al.*, 1978; Drake, 1982b), it has recently been reported that *Clostridium scatologenes*, previously not known to be an acetogen, contains carbon monoxide dehydrogenase and is capable of acetogenic growth on H₂/CO₂ and CO/CO₂ (Küsel *et al.*, 2000), suggesting that certain organisms previously not identified as acetogens might nonetheless be capable of engaging the acetyl-CoA pathway and growing acetogenically.

Acid tolerance among the clostridia has not been extensively evaluated and there are only a few known clostridia that grow at low pH. *Clostridium botulinum* can grow and produce toxin at pH 4.0 (Raattjes & Smelt, 1979) and certain strains of *Clostridium tyrobutyricum* appear to be capable of growth at pH 4.1 (Gibson, 1965). Several clostridial isolates (*Clostridium butyricum*, *Clostridium saccharobutyricum* and *Clostridium acetobutylicum*) obtained from an acidic gleyish bog soil grow at pH 4 (Hammann & Ottow, 1976). A mutant strain of *Moorella thermoacetica* (formerly *Clostridium thermoacetica*) grows at pH 4.5 (Schwartz & Keller, 1982). The thermophile '*Clostridium thermoamylolyticum*' RB-1 (DSM 2335), which exhibits a 98–99% 16S rRNA sequence similarity to that of *Thermoanaerobacterium* species, grows at pH 3.5 and is the only previously described species of *Clostridium* capable of growth below pH 4 (Collins *et al.*, 1994; DSMZ catalogue of strains, 1998). Other acid-tolerant bacteria within clostridial cluster I are the anaerobic bacteria *Sarcina maxima* and *Sarcina ventriculi*, that grow at a pH as low as pH 2 (Canale-Parola, 1986).

Although some of the acid-tolerant clostridia are able to fix N₂, none of these bacteria are known to fix N₂ at low pH. Current evidence suggests that N₂ fixation is negligible in acidic forest soils (Hendrickson, 1990; Limmer & Drake, 1996). That N₂ fixation increases in acidic forest soils after liming (Nohrstedt, 1988; Limmer & Drake, 1996) suggests that organisms capable of fixing N₂ are present in acidic soils and that N₂ fixation by such organisms is impaired by acidic conditions. The results of the present study suggest that previously uncharacterized clostridial species reside and might fix N₂ in the anoxic microzones of acidic forest soil and litter.

Description of *Clostridium akagii* sp. nov.

Clostridium akagii (a.ka'gi.i. L. gen. n. *akagii*, named after James M. Akagi, who worked for more than three decades at the University of Kansas on the physiology of various anaerobic bacteria).

Cells are rods, 2–11 × 1 µm, motile, have 10–15 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. Anaerobic with a chemo-organotrophic, fermentative metabolism. Grows on glucose, cellobiose, xylose, arabinose, maltose, mannose and salicin. Glucose is fermented to acetate, butyrate, lactate, H₂ and CO₂; under certain conditions, formate is also formed. Does not grow on mannitol, lactose, sucrose, glycerol, melezitose, raffinose, rhamnose, sorbitol, trehalose, H₂/CO₂, CO/CO₂, vanillate, Casamino acids, peptone or various purines and pyrimidines. Does not reduce nitrate or sulfate. Fixes N₂ at pH 3.7. Grows at 5–30 °C with an optimum at 20–25 °C. Grows at pH 3.7–7.1 with no distinct optimum between pH 4.2 and 6.8. At pH 4 and

25 °C, the doubling time on glucose approximates 11 h. Catalase, oxidase, tryptophanase and urease negative. Cells do not contain cytochromes. G+C content is 31.4 mol%. The type strain, CK58^T, was isolated from acidic beech litter and has been deposited at the DSMZ as DSM 12554^T.

Description of *Clostridium acidisoli* sp. nov.

Clostridium acidisoli (a.ci.di.so'li. L. adj. *acidus* acidic; L. neut. n. *solum* soil; M.L. gen. neut. n. *acidisoli* of acidic soil).

Cells are rods, 3–7 × 1 µm, motile, have 8–12 peritrichous flagella, are spore-forming and stain Gram-positive. Cultures contain single cells and cell chains; cells in cell chains are not separated after septum formation. Anaerobe with a chemo-organotrophic, fermentative metabolism. Grows on glucose, cellobiose, xylose, arabinose, maltose, mannose, salicin, mannitol, lactose, sucrose, glycerol, melezitose, raffinose and rhamnose. Glucose is fermented to acetate, butyrate, lactate, H₂ and CO₂; under certain conditions, formate is also formed. Does not grow on sorbitol, trehalose, H₂/CO₂, CO/CO₂, vanillate, Casamino acids, peptone or various purines and pyrimidines. Does not reduce nitrate or sulfate. Fixes N₂ at pH 3–7. Grows at 5–37 °C with an optimum at 25–30 °C. Grows at pH 3.6–6.9 with no distinct optimum between pH 3.6 and 6.6. At pH 4 and 30 °C, the doubling time on glucose approximates 3.5 h. Catalase, oxidase, tryptophanase and urease negative. Cells do not contain cytochromes. G+C content is 30.7 mol%. The type strain, CK74^T, was isolated from acidic peat-bog soil and has been deposited at the DSMZ as DSM 12555^T.

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