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EXPERIMENTAL ARTICLES

Clostridium alkalicellum sp. nov., an Obligately Alkaliphilic Cellulolytic Bacterium from a Soda Lake in the Baikal Region

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Abstract—The first anaerobic alkaliphilic cellulolytic microorganism has been isolated from the Verkhnee Beloe soda lake (Buryatiya, Russia) with pH 10.2 and a salt content of up to 24 g/l. Five strains were characterized. Strain Z-7026 was chosen as the type strain. The cells of the isolate are gram-positive spore-forming rods. A mucous external capsule is produced. The microorganism is obligately alkaliphilic, growing in a pH range of 8.0–10.2, with an optimum at pH 9.0. Sodium ions and, in carbonate-buffered media, sodium chloride are obligately required. The microorganism is slightly halophilic; it grows at 0.017–0.4 M Na^+ with an optimum at 0.15–0.3 M Na^+. The metabolism is fermentative and strictly anaerobic. Cellulose, cellobiose, and xylan can be used as growth substrates. Plant and algal debris can be fermented. Lactate, ethanol, acetate, hydrogen, and traces of formate are produced during cellulose or cellobiose fermentation. Yeast extract or vitamins are required for anabolic purposes. The microorganism fixes dinitrogen and is nitrogenase-positive. It is tolerant to up to 48 mM Na_2S. Growth is not inhibited by kanamycin or neomycin. Chloramphenicol, streptomycin, penicillin, ampicillin, ampiox, bacillin, novobiocin, and bacitracin suppress growth. The DNA G+C content is 29.9 mol %. According to the nucleotide sequence of its 16S rRNA gene, strain Z-7026 is phylogenetically close to the neutrophilic cellulolytic bacteria *Clostridium thermocellum* (95.5%), *C. aldrichii* (94.9%), and *Acetivibrio cellulolyticus* (94.8%). It is proposed as a new species: *Clostridium alkalicellum* sp. nov.

Key words: soda lakes, alkaliphiles, anaerobes, cellulose decomposition, dinitrogen fixation, clostridia.

Cellulose is the most widespread carbohydrate polymer on Earth; green algae and higher plants are its main producers. Cellulolytic microorganisms, both fungal and bacterial, mostly occupy "normal" habitats where green plants also grow [1]. Decomposition of masses of plant debris rapidly results in establishment of anaerobic conditions. Anaerobic thermophilic bacteria that cause self-heating of piles of plant debris (compost) are a group characteristic of extreme conditions. Among these species, *Clostridium thermocellum* [2, 3] and *C. stercorarium* [4], with growth optima at 60–65°C, have been studied in detail. In hydrothermal vents, allochthonous cellulose is decomposed by *Caldicellulosiruptor saccharolyticum* [5] (=*Anaerocellum thermophilum* [6]). Fungi and aerobic bacteria predominate under acidic conditions; anaerobic cellulose decomposition has not, however, been observed at pH < 6 [1]. In marine hypersaline lagoons, the mass of green algae is decomposed by the extreme haloanaerobe *Halocella cellulolytica* [7]. In spite of the high taxonomic diversity of the cellulolytic bacteria isolated, alkaliphilic forms, either aerobic or anaerobic, were unknown and soda lakes were not considered as possible habitats of cellulolytic bacteria in reviews on microbial utilization of cellulose [1, 8–10].

Soda lakes are typical terrestrial habitats, and their microbial communities may be considered as analogues of ancient terrestrial communities [11]. They have connections with the flora of the surrounding landscapes, which provide a possible source of allochthonous cellulose. Plant debris is actively decomposed in soda lakes. This process was first described by B.L. Issatchenko in the lakes of the Kulundin Steppe in the 1930s [11]. With the onset of a systemic approach to investigation of the anaerobic communities of soda lakes, cellulose-decomposing organisms were found to be active in the alkaliphilic community (pH 10.2) in the equatorial soda lake Magadi [12] and, subsequently, in eight lakes of Wadi el Natrun, which have a salt content of 200–350 g/l. While cellulose was found to be actively decomposed by the microbial communities of these lakes at 1.5 M Na^+, 3 M Na^+ was the critical concentration (Zhilina, unpublished data).

Investigations of the process of cellulose decomposition in situ in aerobic and anaerobic zones of the low-mineralization soda lakes of the Baikal region have

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been performed in the laboratory headed by B.B. Namsaraev (Institute of General and Experimental Biology, Siberian Division, Russian Academy of Sciences). High numbers of cellulolytic bacteria were found [13]. The sequential stages of anaerobic cellulose decomposition were studied for the communities of two central Asian soda lakes, Hadyn (Tuva) and Verkhnee Beloe (Baikal region), with pH 10 and a salt content of about 30 g/l [14]. These stages include cellulose hydrolysis, development of saccharolytic dissipotrophs, and syntrophic decomposition of fermentation products by hydrogenotrophic sulfate-reducing bacteria [11, 15]. From these communities, saccharolytic non-spore-forming bacteria of various phylogenetic groups were isolated: *Spirochaeta asiatica* [16], the low G+C gram-positive *Anoxynatronum sibiricum* [17] and *Alkalibacter saccharofermentans* [18], and a representative of the *Cytophaga–Flavobacterium–Bacteroides* group, *Alkaliflexus imshenetskii* [19]; none of them was capable of cellulose utilization. Only *Alkaliflexus*, isolated on cellobiose, exhibited very weak cellulolytic activity. The isolation of the cellulolytic component of the system was necessary for understanding the initial segment of the trophic chain of the anaerobic alkaliphilic community.

In the present study, we report the isolation and provide a characterization of the first alkaliphilic cellulolytic bacterium isolated from the cellulose-decomposing community of the Verkhnee Beloe soda lake. We suggest that this organism be assigned to a new species of the genus *Clostridium*: *C. alkalicellum.*

MATERIALS AND METHODS

Source of isolation. The study was performed with sample no. 27, collected by T.N. Zhilina in 1995 from a coastal lagoon of the Verkhnee Beloe Lake, which has a pH of 10 and mineralization of 7.5 g/l. The sample consisted of an upper cyanobacterial film (2–3 mm) with underlying anaerobic sludge, colored black as a result of sulfate reduction.

Cultivation conditions. In order to obtain enrichment cultures, 1 g of the sludge was mixed anaerobically with 100 ml of liquid medium, and 1 ml of this mixture was used to inoculate 10 ml of carbonate-buffered (pH 10) mineral medium imitating the chemical composition of the lake water, as previously described in [14]. Cellulose (Whatman no. 1) or microcrystalline cellulose (MCC) Sigmacell 101 (Sigma, United States) were added as the substrates. Pure cultures were maintained on an optimized medium of the following composition (g/l): KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; NH_4Cl, 0.5; KCl, 0.2; NaCl, 1.0; Na_2CO_3, 2.6; $NaHCO_3$, 19.0; $Na_2S \cdot 9H_2O$, 0.5; yeast extract, 0.2; cellulose, 1.0–3.0; trace element solution [14], 1 ml/l; and resazurin, 0.001 (pH 8.8). Cellobiose and the other carbohydrates tested were introduced into the alkaline medium immediately before inoculation in the form of concentrated sterile aqueous solutions; the final concentration was 3.0 g/l. Both preparation of the medium and incubation were performed under strictly anaerobic conditions in a nitrogen atmosphere.

Pure cellulolytic cultures were isolated from consortia by culture transfers using end-point tenfold dilutions in a medium with MCC and subsequent isolation of the colonies on agarized medium. Strains Z-7026, Z-7022, Z-7021, Z-7024, and Z-7023 were obtained in the form of pure cultures and characterized; strain Z-7026^T was chosen for detailed study.

Physiological characteristics. Electron acceptors were added as concentrated solutions to a sterile medium devoid of NH_4Cl and containing 0.5 g/l of thioglycollate as the reducing agent; their final concentrations (mM) were $Na_2S_2O_4$, 1.0; Na_2SO_3, 2.0 and 10.0; $NaNO_2$, 2.0 and 10.0; $NaNO_3$, 20.0; $Na_2S_2O_3 \cdot 5H_2O$, 20.0; Na_2SO_4, 20.0; and S^0, 2% (wt/vol). Utilization of sulfur-containing electron acceptors was judged from the production of hydrogen sulfide. Nitrite reduction was determined with the Griess reagent.

Nitrogenase activity was assessed on the optimal medium devoid of a nitrogen source except for the N_2 present in the gas phase. The strains were grown anaerobically on a cellobiose medium in Hungate tubes containing 5 ml of medium and a 11.5-ml gas phase. After four days of growth, 10% acetylene was added, and ethylene was determined chromatographically after one day of incubation in the dark at 35°C using a model 3700 gas chromatograph (Russia). At the end of the incubation period, protein was determined by the Lowry method.

To study the effect of pH, the required pH values were obtained by titration of the medium with 10% HCl or 10% NaOH. The sodium carbonate in the medium was replaced by sodium bicarbonate at a tenfold lower concentration; the sodium concentration was adjusted by adding NaCl.

The effect of sodium carbonates was investigated by replacing them with equimolar amounts of NaCl. The pH was maintained at a value of 9.0 with 50 mM serine buffer. In order to determine the NaCl requirement, it was substituted with an equimolar amount of sodium carbonate and bicarbonate. The other chlorides present in the medium were substituted with sulfates. The effect of temperature was investigated within the range 18–60°C at optimal values of pH and sodium chloride concentration.

Two approaches were used to determine the resistance of the strains to drying. In the first variant, filter paper was soaked in the culture, dried for one day under aerobic conditions, and transferred to an anaerobic medium to monitor cellulose decomposition. In the second variant, the culture was dried gradually for 1.5 months under a cotton plug at 55°C until a dry crystal precipitate was obtained. After 50 days of storage, anaerobic media with Whatman no. 1 paper and MCC were inoculated with the culture imbedded in the salt precipitate, and cellulose decomposition was monitored.

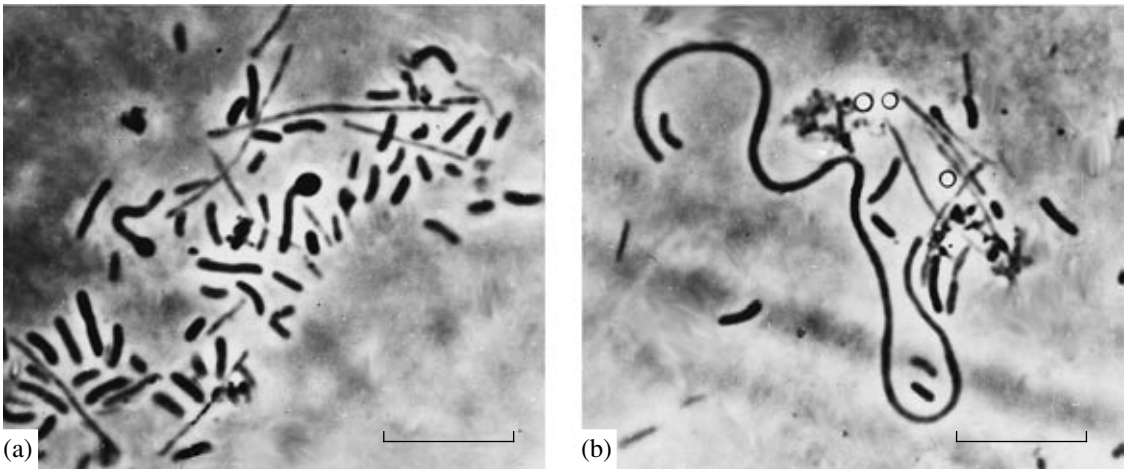

Fig. 1. Bacterial morphology in cellulolytic consortium 7012. The bar is 10 μm.

Tolerance to sulfide was determined in the optimal medium with cellobiose or MCC, with sodium thioglycollate added as the reducing agent. A sterile 25% solution of $Na_2S \cdot 9H_20$ was injected into test tubes with 10 ml of medium and N_2 prior to inoculation; the final sulfide concentrations were 2 and 10–100 mM, with a step of 10 mM. In order to correct for the resulting pH values and sulfide concentrations, an uninoculated control series was used.

Analytical techniques. Growth on cellobiose was monitored by measuring OD_{600} in Hungate tubes using a Specol-10 spectrophotometer (Jena, Germany). Hydrogen was determined using an LKhM-80 gas chromatograph equipped with a katharometer and a column filled with a 5 Å molecular sieve. Organic fermentation products were identified using a Stayer HPLC chromatograph equipped with a refractometric detector and an Aminex HPX-87H column (Bio-Rad, United States); 5 mM H_2SO_4 was used as the eluting agent. Cellobiose was determined using a reaction with phenol. Formate was determined colorimetrically. Dissolved sulfide was determined colorimetrically from methylene blue reduction according to the procedure described in [17]. The catalase test was performed by mixing a drop of biomass with a drop of 3% hydrogen peroxide on a slide. The formation of gas bubbles indicated the presence of catalase.

Morphology. Live cells were studied using a ZETOPAN phase microscope (Austria). Ultrathin sections, prepared as previously described in [16], and cells stained with 1% phosphotungstic acid to reveal flagella were examined using a JEM-100C electron microscope (Jeol, Japan).

DNA analysis. The DNA G+C content was determined from the melting temperature, and DNA homology was determined by the optical reassociation method described in [17].

Determination of the 16S rRNA gene sequence. Amplification and sequencing of 16S rRNA gene fragments were performed according to the method described in [17]. The nucleotide sequences of the 16S rDNA of strain Z-7026 were manually aligned with the corresponding sequences of the known species of cluster III clostridia using the BIOEDIT sequence editor (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic tree was constructed using various algorithms implemented in the TREECON software package (http://bioc-www.uia.ac.be/u/yvdp/treeconw.html), with *Rhodococcus ruber* as the outgroup organism.

The 16S rDNA sequence of strain Z-7026 was deposited with GenBank under the accession number AY959944.

RESULTS

Isolation. The anaerobic alkaliphilic microbial community from the Verkhnee Beloe soda lake decomposed filter paper and MCC relatively rapidly, during which the substrate turned yellow. In the common mucous stockinglike envelope, spore-forming rods of various morphotypes, attached to cellulose particles by one of the poles, and cytophagas spreading over the particle surface were microscopically discernable. After pasteurization at 90°C for 20 min, "consortium 7012" was obtained, consisting of a minimal number of forms; this consortium actively decomposed paper and turned it yellow. The consortium consisted of spore-forming and non-spore-forming, but heat-resistant, bacteria. Morphologically different rod-shaped cells were present, some of them with spores (Figs. 1a, 1b).

In the serial dilutions, cellulose decomposition occurred until 10^{-7}. The MCC turned yellow until a 10^{-5} dilution, and, in the 10^{-6} and 10^{-7} dilutions, it remained white. Inoculations of agarized medium with MCC were made from the last dilution using the Hungate roll-tube technique. After 20 days, the colonies were transferred to liquid media with cellobiose, MCC, or filter paper using glass capillary tubes. Some of the

Table 1. Characteristics of alkaliphilic cellulolytic anaerobes from the consortium of Verkhnee Beloe Lake

Strains	G+C, mol %	DNA–DNA homology with Z-7026^T, %	Sliming of MCC	Growth with cellobiose	Cell morphology	Motility
Z-7026^T	29.9	100	+	+	Straight or slightly curved rods	–
Z-7021	29.9	97	–	+	Short straight rods	+
Z-7022	30.2	97	–	±	″	+
Z-7023	29.9	98	+	±	Straight or slightly curved rods	–
Z-7023^1	29.9	None	+	+	″	–
Z-7024^1	30	95	–	+	Short straight rods	+

Note: "±," weak growth; "+," the feature is present; and "–," the feature was not observed. DNA–DNA hybridization revealed the following relationships in the strain pairs: Z-7022–Z-7021, 97%, and Z-7023^1–Z-7023, 98%.

colonies grew only with cellobiose, others, also on MCC and filter paper. The cellulose undergoing decomposition remained white, without the yellowing characteristic exhibited under decomposition by the consortium. This result indicates the presence of additional cellulolytic bacteria in the consortium.

The isolated cellulose-decomposing strains, although somewhat different phenotypically, were genotypically close, as indicated by their DNA G+C content and their DNA–DNA homology (95–98%); these results suggested their affiliation to one species (Table 1). Strain Z-7026 was investigated as the type strain.

Cultural properties. The morphology of the Z-7026 cells varied somewhat depending on the substrate and age of the culture. The cells of a young culture were short, straight or slightly curved rods 1.1–2.5 μm long and 0.5–0.7 μm thick. Binary division occurred by constriction, and there was no septum formation. On cellulose, the cells were mostly single or occurred in pairs with a total length not exceeding 3.75 μm. They attached to the fibers at right angles, forming a brushlike structure (Fig. 2a). After destruction of the aggregates, free cells appeared (Fig. 2b). On cellobiose, the cells were single or in pairs or chains, sometimes up to 25 cells long. The cells were often positioned at an angle to each other (Fig. 2d). Some cells had a length of 5–7 μm (Figs. 2d, 2f), and long filaments occurred in old cultures.

Type strain Z-7026 was nonmotile and no flagella were revealed, although, in some strains, motility was recorded, and one or two subterminal flagella were found (Table 1). The presence of thin fimbria was revealed (Figs. 2e, 2f). Sliming of cellulose and MCC occurred during their dissolution by strain Z-7026. A mucous capsule was revealed by staining with phosphotungstic acid; it was most pronounced in the cellulose-grown cells (Fig. 2c). Positive staining with Alcian Blue indicated the presence of acid polysaccharides in the capsule. The Gram reaction was positive. Ultrathin sections showed the gram-positive structure of the cell wall. It consisted of two osmiophilic layers and an external mucous capsule with no outer membrane (Figs. 3a, 3b). The cells did not lyse in distilled water or in a solution of sodium dodecyl sulfate and were resistant to lysozyme. Hexagonal inclusions, possibly reserve compounds, were found in the cytoplasm (Figs. 3a, 3b). Staining with Lugol's reagent resulted in brown coloration of the cell interior, characteristic of reserve polysaccharides.

When grown on MCC, strain Z-7026 formed circular flat colonies 0.5–1 mm in diameter, with a yellowish center and a whiter granular edge. On cellobiose, the colonies were white, flat, and dense, had a smooth edge, and were 1–2 mm in diameter.

The organism is spore-forming. The spores are round, 0.7–1.0 μm in diameter; they occur close to one of the cell poles (Figs. 2a, 2b). According to the results of incubation for 5, 10, 15, 20, and 30 min at 80, 90, and 100°C, the culture should be considered moderately heat-resistant. Neither the cells nor the spores survived a 5-min heating at 100°C; however, after a 30-min pasteurization at 80°C, growth and cellulose decomposition resumed. Growth and MCC decomposition were observed in an anaerobic medium inoculated with an MCC culture air-dried for 1.5 months at 55°C.

Growth characteristics. Strain Z-7026 is an obligate extremely alkaliphilic organism with a pH growth range of 8.0–10.2 and an optimum at pH 9.0. No growth or cellulose decomposition occurred at pH 7.5 or lower or at pH 10.5 or higher. The organism is mesophilic, with the growth optimum at 35–40°C. No growth occurred at 45°C. The upper growth limit is 42°C, and the lower one, 18°C. When the organism is grown on alkaline media with sodium carbonates, sodium chloride is also required; it is, therefore, moderately haloalkaliphilic, with the growth range 0.017–0.4 M Na^+ and the growth optimum at 0.15–0.3 M Na^+. No growth occurred at 0.46 M Na^+. Sodium chloride could not be replaced by carbonate salts providing equimolar sodium concentrations. Under optimal conditions, the doubling time on cellulose was 14 h.

Strain Z-7026 is strictly anaerobic and does not use oxygen as an electron acceptor. It is catalase-negative. No aerobic growth was observed, regardless of the

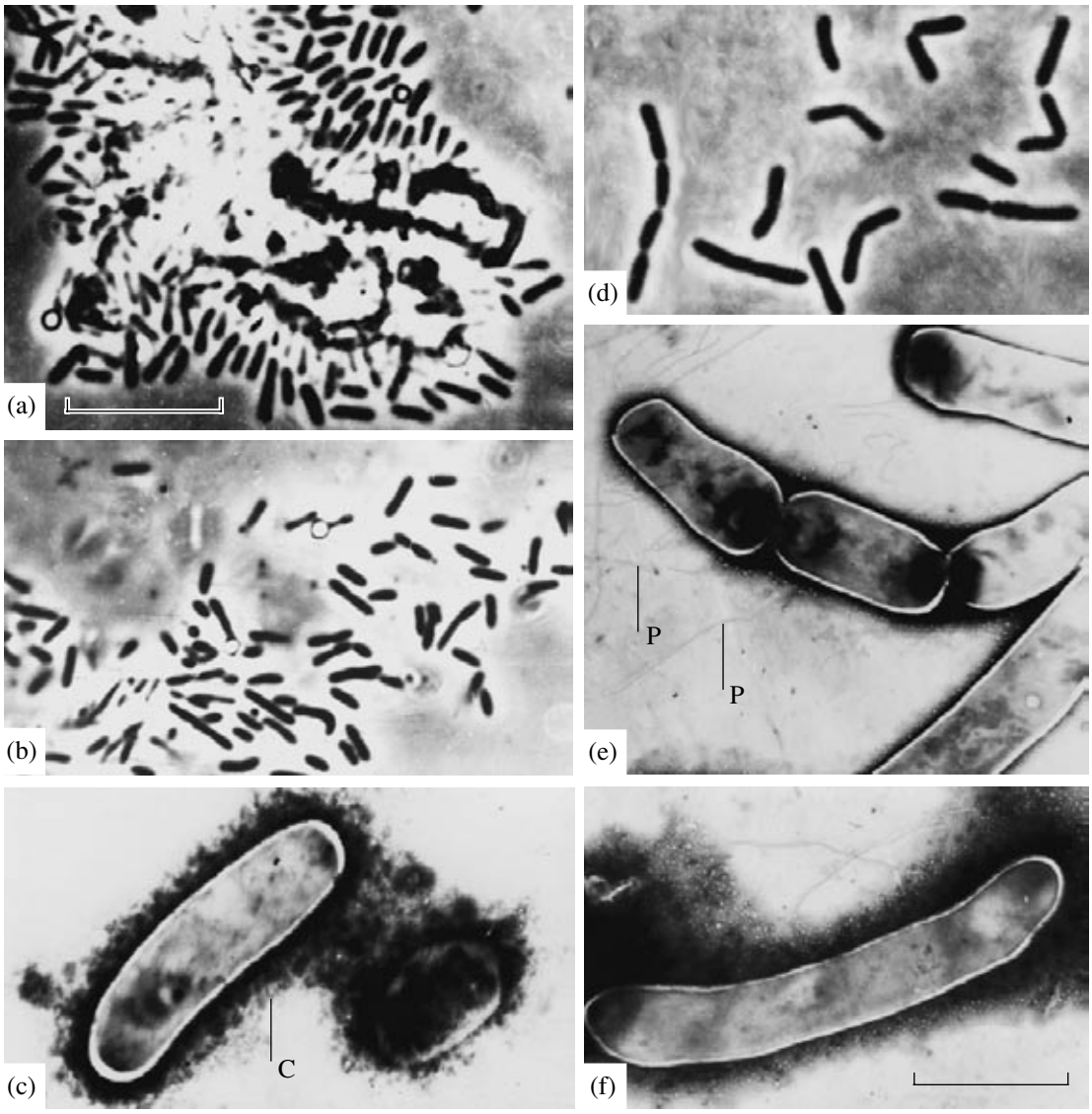

Fig. 2. Whole cells of strain Z-7026. (a, b, d) Light microscopy (bar, 10 μm). (c, e, f) Electron microscopy (bar, 1 μm). (a, b, c) Growth on MCC; (d, e, f) growth on cellobiose. C, mucous capsule; P, pili.

thickness of the layer of liquid medium. A reducing agent was required; sodium sulfide and thioglycollate were equally acceptable. In the absence of a reducing agent, weak growth (25% of that observed under the optimal Na_2S concentration of 2 mM) occurred only in the first transfer but not in the subsequent ones. The culture was extremely tolerant to sulfide (up to 48 mM). The best growth was observed at 2 mM Na_2S; at other concentrations, it decreased approximately twofold. At high sulfide concentrations, the pH remained within the range 9.0–9.5 and, thus, could not impede growth.

Strain Z-7026 was found to be a highly specialized cellulose decomposer. It did not utilize monosaccharides, and cellobiose was the only disaccharide utilized. Yeast extract or vitamins were required for anabolism. The organism did not utilize xylose, glucose, mannose, fructose, sorbitol, sucrose, maltose, lactose, rhamnose, trehalose, fucose, galactose, inositol, ribose, arabinose, sorbose, starch, potato dextrin, dextran, glycogen, casamino acids, acetyl-D-glucosamine, glycerol, galactomannan, pullulan, gum arabic, alginic acid, xanthan, peptone, yeast extract, agar, chitin, ethanol, lactate, pyruvate, acetate, formate, butyrate, propionate, glycolate, fumarate, or betaine.

Under optimal growth conditions, complete hydrolysis of cellulose of various types (Whatman no. 1 paper, Whatman CF/F, Avicel, MCC of various brands) provided at a concentration of 20 mg/10 ml occurred in seven days. Since the isolate hydrolyzed cellulose at approximately the same rate as the original enrichment, microbial interactions in the consortium did not enhance cellulose decomposition.

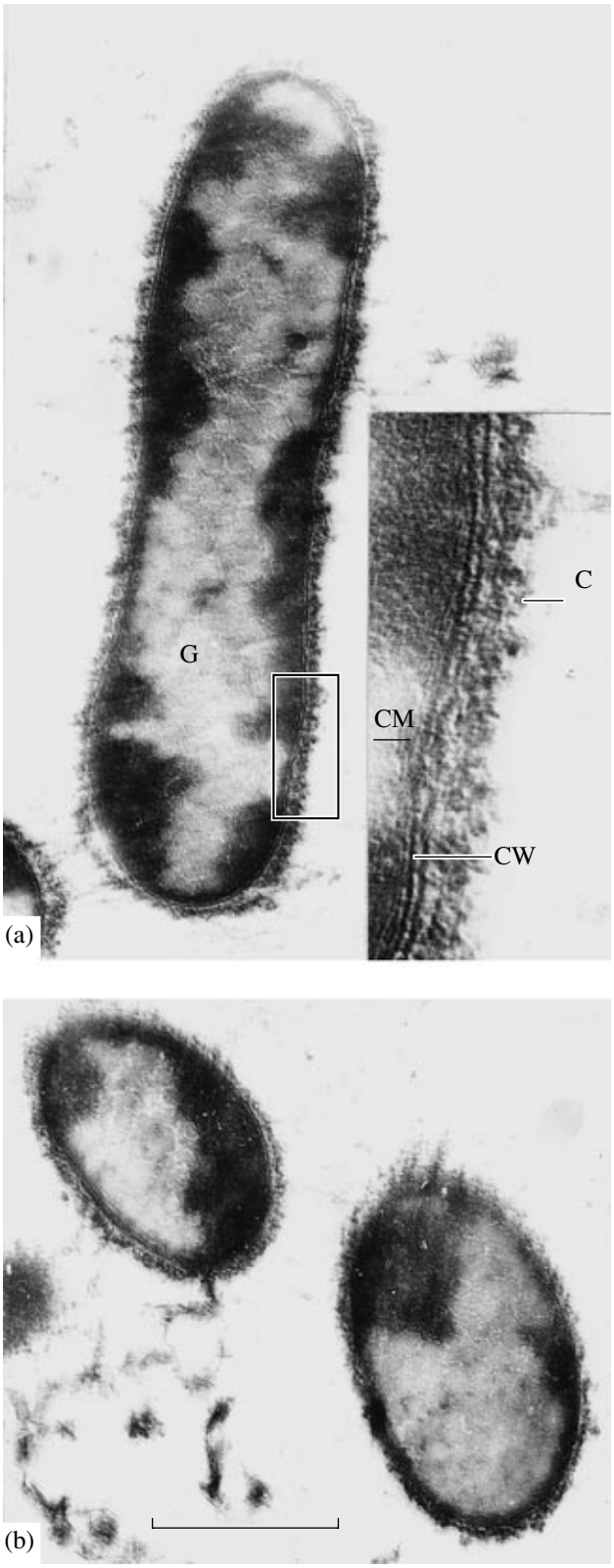

Fig. 3. Ultrathin sections of strain Z-7026 cells: (a) longitudinal section of a cell; the insert shows a fragment of the cell wall at a higher magnification (70000×); (b) cross cell sections. The bar is 0.5 μm. C, mucous capsule; CW, cell wall; CM, cytoplasmic membrane; G, polysaccharide granules.

Growth on carboxymethylcellulose was weak. Xylan was among the polymeric substrates utilized by the strain. The organism was capable of hydrolyzing matgrass (*Nardus stricta*) straw and the algal biomass of *Cladophora sivashensis*; 200 mg/10 ml medium of dry biomass was completely decomposed in a month. Cyanobacterial biomass was not hydrolyzed.

Hydrogen, acetate, ethanol, and lactate were the main products of cellobiose and cellulose fermentation; among the minor products, formate was present. Three unidentified peaks were revealed by chromatography. From 6.5 mmol of consumed cellobiose, 11.5 mmol lactate, 2.1 mmol acetate, 6.5 mmol ethanol, 1.4 mmol formate, and 6.6 mmol hydrogen were produced. This formula gives a 68.5% carbon balance and a 93.2% hydrogen balance, the hydrogen balance being more informative, since quantification of the carbon dioxide produced in the course of fermentation is impossible for organisms grown with a carbonate buffer.

The metabolism was strictly fermentative. Neither sulfur compounds (SO_4^{2-}, $S_2O_3^{2-}$, SO_3^{2-}, $S_2O_4^{2-}$, or S^0) nor nitrate and nitrite could act as electron acceptors. Nitrite completely inhibited growth at 2 mM. Other electron acceptors did not affect growth.

Ammonium and nitrate did not inhibit growth. Since the organism was able to survive numerous transfers in the absence of nitrogen sources other than N_2 in the gas phase, nitrogenase activity was investigated. The cells of five strains (Z-7026^T, Z-7022, Z-7021, Z-7024, and Z-7023) proved able to reduce acetylene to ethylene when grown on a medium with cellobiose and 200 mg/l of yeast extract or 10 ml/l of Wolin vitamin solution in an atmosphere of 100% N_2 without ammonium nitrogen. In the presence of yeast extract, the acetylene reduction rate was 1–5 nmol C_2H_4/h per ml of culture (88–183 μg of protein) depending on the strain. For strain Z-7026^T, the rate was 3.6 nmol C_2H_4/h per ml of culture (143.6 μg of protein). This value was twice as high on the medium containing vitamins: 5–10 nmol C_2H_4/h per ml of culture (110–183 μg of protein). For strain Z-7026^T, the rate was 10.6 nmol C_2H_4/h per ml of culture (128.6 μg of protein). Growth with N_2 as the sole nitrogen source and the presence of nitrogenase testify to the ability of the investigated strains to fix dinitrogen.

Antibiotics possessing different spectra of action (chloramphenicol, streptomycin, penicillin, ampicillin, ampiox, bacillin, novobiocin, and bacitracin) completely suppressed the growth of strain Z-7026 at concentrations of 100 mg/l. The organism was highly resistant to kanamycin, with growth comparable to the control, and partially resistant to neomycin, which suppressed growth by 50% as compared to the control.

Analysis of the 16S rRNA genes. Partial sequencing of the 16S rRNA gene of strain Z-79026 was performed (1343 nucleotides corresponding to *Escherichia coli* positions 60–1418). Phylogenetic analysis

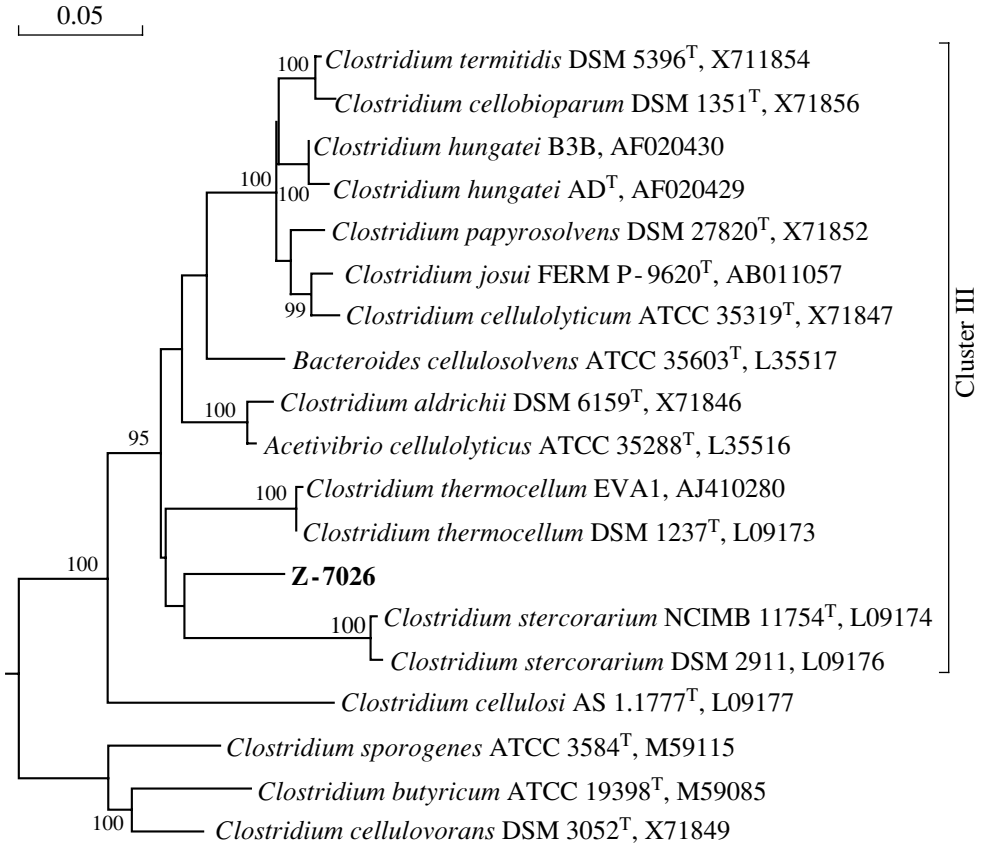

Fig. 4. Phylogenetic position of strain Z-7026 in the phylogenetic tree of clostridial cluster III of the low-G+C lineage of gram-positive bacteria. The tree was constructed based on comparative analysis of 16S rRNA gene sequences. The scale bar corresponds to 5 nucleotide substitutions per 100 nucleotides.

indicated that the strain belonged to the clostridia of phylogenetic cluster III [20], forming an independent branch among the other cellulolytic and noncellulolytic neutrophilic clostridia (Fig. 4). Among the species of cluster III for which 16S rDNA sequences are available, strain Z-7026 was closest to *Clostridium thermocellum* (95.5%), *C. aldrichii* (94.9%), and *Acetivibrio cellulolyticus* (94.8%). The homology between the 16S rDNA sequences of strain Z-7026 and those of other clostridia of phylogenetic cluster III did not exceed 92%. Thus, phylogenetic analysis showed that strain Z-7026 should be classified as representing a new species-rank taxon.

DISCUSSION

Strain Z-7026, together with the other strains of the new species, is the first alkaliphilic cellulolytic anaerobe. The capacity of an organism for active hydrolysis of cellulose under alkaline conditions is a result of the presence of respective cellulases. In a parallel investigation [21], we demonstrated the production by strain Z-7026 of extracellular enzymes of the cellulase complex with a pH optimum at 8.0–8.5; over 70% of the maximum activity was retained at pH 9.2.

The organism was found to be a highly specialized cellulose decomposer: out of the products of cellulose hydrolysis, only cellobiose was utilized. As for natural cellulose-containing plant materials, the straw of *Nardus stricta* and the biomass of the green alga *Cladophora* were actively hydrolyzed and supported growth. Matgrass was more easily decomposed, and the growth was better. In its natural habitat, the organism probably utilizes exogenous plant material rather than the autochthonous dead algal biomass.

The capacity to hydrolyze cellobiose often occurs in parallel with the capacity to hydrolyze xylan, another carbohydrate polymer of plant cell walls. It is utilized by a number of anaerobic cellulolytic clostridia. Strain Z-7026 grows with xylan. When this organism was grown with cellobiose, xylanases were produced. Their optimum was, however, in a more acidic range than that of cellulases [21]. In spite of the presence of xylanase, strain Z-7026 does not utilize pentoses, unlike, for example, *C. stercorarium* [4].

Cellulolytics belong to the initial segment of the trophic chain. In a cooperative microbial community, they supply substrates for dissipotrophic bacteria capable of utilizing the products of cellulose hydrolysis: cellobiose, cellodextrines, and glucose. The gliding

cytophaga *Alkaliflexus* [19] isolated from this community mostly utilizes cellobiose. It has weak hydrolytic activity; however, the isolated strains failed to grow on cellulose, although microscopic examinations showed that such organisms constitute a significant portion of the microbial consortium surrounding the cellulose particles. The metabolic products of cellulolytics can be used by other components of the community, particularly sulfate-reducing bacteria [11, 14, 15].

Since our new isolates, like many other cellulose-utilizing anaerobic bacteria, are capable of N_2 fixation [8, 22, 23], they do not require bound nitrogen. The values of acetylene reduction rates obtained for the new isolates are comparable to those of other dinitrogen-fixing bacteria, e.g., isolates from strongly saline takyr-like soils such as *Xanthobacter flavus* and *Alcaligenes paradoxus* [23]; however, the values are lower than that characteristic of *C. hungatei* [22] isolated from soil. Nevertheless, in its natural habitat, the new alkaliphilic cellulolytic can supply other members of the anaerobic community with bound nitrogen. Due to its specificity with respect to cellulose, a substrate unavailable to other anaerobes, the new cellulolytic bacterium is, to a certain extent, nutritionally independent. However, the organism cannot be completely autonomous because of its vitamin requirements.

In the presence of sulfates, cellulose decomposition in the anaerobic zone of the alkaliphilic community terminates with sulfidogenesis [14], the most important process of the terminal stage of organic matter degradation in soda lakes [24]. Strain Z-7926 was found to be highly tolerant to sulfide and, thus, capable of growth within a sulfidogenic community. The physiological parameters of strain Z-7026 growth, such as the ranges of pH, temperature, and salinity, are in good accord with the conditions of its natural habitat. The organism obligately requires sodium. When grown in alkaline media with sodium carbonates at high pH values, it also requires sodium chloride. This sodium chloride requirement indicates that this moderately halophilic organism is probably of external origin and became adapted to the new environment. Its spores retain viability after aerobic drying and can survive aerial transportation. They can survive in dried brines after drying out or freezing of shallow soda lakes under the conditions of the cryoarid climate common to the southwestern Baikal region.

The taxonomic position of the organism should be determined based on both its phenotypic and phylogenetic characteristics. Strain Z-7026 is a gram-positive strict anaerobe producing endospores. Together with the low G+C content, these phenotypic characteristics determine its placement in the genus *Clostridium* [3]. According to the analysis of the 16S rRNA genes, strain Z-7026 is related to *Clostridium thermocellum* [2, 3], *C. aldrichii* [25], and *Acetivibrio cellulolyticus* [26], as well as to other cellulolytic clostridia of cluster III (Fig. 4). The level of homology between the 16S rDNA sequences of strain Z-7026 and the species of cluster III do not, however, exceed 95.5%, which is significantly lower than the 97% threshold accepted for the strains of one species. These results indicate that strain Z-7026 does not belong to any known species and should be described as an individual species. Its DNA G+C content differs by 8–10 mol % from that of its relatives. Since strain Z-7026 requires sodium ions, develops in an alkaline pH range, and dwells in a specific habitat, it is substantially different with respect to its phenotype from all the three related species (Table 2). It differs from *C. thermocellum* by its inability to grow at elevated temperatures; the shape of its spores; and its inability to utilize glucose, esculin, amygdalin, or salicin. It differs from *C. aldrichii* by having a more limited set of fermentation products, an inability to utilize esculin, and a spherical spore shape. It is distinguished from *A. cellulolyticus* by cell morphology, lack of motility, presence of endospores, occurrence of lactate among the fermentation products, and inability to utilize salicin (Table 2).

Based on the above-mentioned phenotypic and genotypic differences and the results of the phylogenetic analysis, it is proposed that the organism be assigned to a new species, *Clostridium alkalicellum* sp. nov. with the following description.

Description of *Clostridium alkalicellum* sp. nov.

Al.ka.li.cel'lum n.N.Lat. *alkali*, from the Arabic adjective *al qaliy*, alkaline; N.L.n. *cellum*, from the Latin noun *cellulose*; N.L.neut.adj. *alkalicellum*, utilizing cellulose under alkaline conditions.

The cells are gram-positive, spore-forming, nonmotile (certain strains are motile due to the presence of flagella), straight or slightly curved rods measuring 0.5–0.7 × 1.1–2.5 μm. Spores are spherical, terminal, 0.7–1.0 μm in diameter, resistant to drying, and moderately heat-resistant. Growth is possible after heating for 30 min at 80°C but not at 100°C.

Colonies grown on medium with MCC are circular, flat, 0.5–1 mm in diameter, and have a yellowish center and a denser white granular edge. On cellobiose, the colonies are white, dense, and flat, with smooth edges, and 1–2 mm in diameter.

Chemoorganoheterotroph. Cellulolytic, utilizing a narrow spectrum of carbohydrate polymers and sugars. Cellulose, cellobiose, and xylan are used as carbon and energy sources. Yeast extract or vitamins are required for anabolism. Capable of decomposing plant and algal debris. Does not utilize monosaccharides or di- and polysaccharides other than the aforementioned ones. Organic acids, alcohols, and protein substrates are not utilized. Products of cellobiose and/or cellulose fermentation are lactate, ethanol, acetate, hydrogen, and traces of formate. Tolerant of up to 48 mM Na_2S in the medium. Grows in the presence of kanamycin and neomycin. Chloramphenicol, streptomycin, penicillin,

Table 2. Characteristics differentiating the new isolate from the phylogenetically close species of cluster III of low-G+C gram-positive bacteria

Characteristics	Z-7026^T	*C. thermocellum* DSM 1237^T [2, 3]	*Acetivibrio cellulolyticus* CD2^T [26]	*C. aldrichii* P-1^T [25]
Morphology	straight or slightly curved rods	straight or slightly curved rods	slightly curved rods	slightly curved rods
Size, μm	0.5–0.7 × 1.1–2.5	0.5–0.7 × 2.5–5	0.5–0.8 × 4–10	0.5–1.0 × 3–5
Gram reaction	+	–	–	+
Motility/flagella	+/–	–	+/one lateral	+/terminal bundles
Endospores/localization	round/terminal	oval/terminal	absent	elongated/subterminal
Temperature, °C, range/optimum	18–42/35–40	>37/60–64	20–40/35	20–45/35
Reaction to salinity	weakly halophilic	freshwater	freshwater	freshwater
pH, range/optimum	8.0–10.2/9.0	ND/7.0	6.5–7.7/7.0	6.2–7.8/7.0
Substrates:				
cellulose	+	+	+	+
cellobiose	+	+	+	+
glucose	–	+	–	–
xylan	+ (xylanase)	– (xylanase)	ND	+
esculin	–	+	–	+
amygdalin	–	+	–	–
salicin	–	+	+	–
Fermentation products	lactate, ethanol, acetate, H_2, traces of formate*	acetate, lactate, ethanol, CO_2, H_2**	acetate, H_2, CO_2, traces of ethanol**	acetate, propionate, butyrate, isobutyrate, isovalerate, lactate, succinate, H_2, CO_2**
Doubling time, h	14	7	ND	ND
G+C, mol %	29.9	38–39	38	40
Source of isolation	anaerobic sludge of a low-mineralization soda lake	anaerobic wastewater fermentor	freshwater methanogenic community	anaerobic wood-processing fermentor

Note: None of the strains utilized starch, lactose, glycogen, sucrose, arabinose, galactose, maltose, melibiose, rhamnose, ribose, or xylose. ND stands for "no data".
* From cellobiose.
** From glucose.

ampicillin, ampiox, bacillin, novobiocin, and bacitracin suppress growth.

Strict anaerobe with a fermentative metabolism. Catalase-negative. Capable of dinitrogen fixation.

Obligate extreme alkaliphile growing at pH 8.0–10.2 with an optimum at pH 9.0. Sodium ions are obligately required. Weak halophile growing at 0.017–0.4 M Na^+ with an optimum at 0.15–0.3 M Na^+. Mesophile, with a growth range from 18 to 42°C and an optimum at 35–40°C. The doubling time under optimal conditions is 14 h.

In different strains, the DNA G+C content varies from 29.9 to 30.2 mol % (29.9 mol % in type strain Z-7026).

Belongs to clostridial cluster III within the lineage of low-G+C gram-positive bacteria. According to the results of 16S rRNA gene sequencing, the closest relatives are *Clostridium thermocellum* (95.5%), *C. aldrichii* (94.9%), and *Acetivibrio cellulolyticus* (94.8%).

Inhabits steppe soda lakes with a moderate salt content, usually with coastal vegetation. The type strain was isolated from the anaerobic cellulose-degrading community of Verkhnee Beloe Lake (Buryatiya, Russia).

The type strain is Z-7026^T (=VKM 2349).

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