Clostridium thermoalcaliphilum sp. nov., an Anaerobic and Thermotolerant Facultative Alkaliphile

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An anaerobic and thermophilic alkaliphile, strain JW/YL23-2^T (T = type strain), was isolated from sewage sludge obtained from a sewage plant in Atlanta, Ga. At pH 10.1 and 50°C, the doubling time of this strain was 19 min. Strain JW/YL23-2^T, a motile rod-shaped bacterium with 2 to 12 peritrichous flagella, exhibited a negative Gram stain reaction but was gram-type positive as judged by the polymyxin B test. No heat-stable (85°C, 15 min) endospores were detected. At 50°C, growth occurred at pH values ranging from 7.0 to 11.0; the optimum pH was 9.6 to 10.1. The temperature range for growth ranged from 27 to 57.5°C; the optimum temperature was 48 to 51°C (pH 10.1). Dissimilatory sulfate reduction was not detected. The organism utilized glucose, fructose, sucrose, maltose, cellobiose, and Casamino Acids. The DNA G+C content was 32 mol% (as determined by chemical analysis). A 16S rRNA sequence analysis revealed a 2% inferred evolutionary distance to *Clostridium paradoxum*. However, the cell wall type of strain JW/YL23-2^T was A4β (L-Orn-D-Asp), while that of *C. paradoxum* was A1τ (m-diaminopimelic acid direct). On the basis of the alkaline pH values and high temperatures for optimal growth, the inability to form spores, and other characteristics different from *C. paradoxum* characteristics, strain JW/YL-23-2 was placed in a new species, *Clostridium thermoalcaliphilum*; JW/YL23-2 (= DSM 7309) is the type strain of this new species.

Most of the alkaliphiles that have been isolated previously are aerobic procaryotes (8), and most of the current knowledge concerning alkaliphiles has been derived mainly from studies performed with aerobic bacteria (8-10, 13). Krulwich and Guffanti (13) defined alkaliphiles as organisms that have an optimum pH for growth of 10.0 or above. Souza et al. (24) isolated a eu(bacterial), alkaliphilic, mesophilic, obligate anaerobe that presumably was a *Clostridium* species. This organism, however, was not well characterized, its description was not validly published, and the organism was not available for comparison. Recently, we described the isolation of a thermophilic, (eu)bacterial, obligately anaerobic alkaliphile, *Clostridium paradoxum* (15). Other than these (eu)bacterial strains, the only anaerobes growing at alkaline pHs that have been isolated previously are alkalitolerant to moderately alkaliphilic archaea (i.e., methanogens [4, 5, 16, 18, 34] and halophiles [23]).

In this paper, we describe the characterization of a second novel thermotolerant obligately anaerobic alkaliphile isolated from the Atlanta Municipal Sewage Plant (Atlanta, Ga.), from which *C. paradoxum* L-15-1 was isolated previously (15). On the basis of characteristics different from the characteristics of *C. paradoxum* and other thermophiles (30), we propose that this strain should be placed in a new species, *Clostridium thermoalcaliphilum*, until the genus *Clostridium* has been redefined.

MATERIALS AND METHODS

Sample collection. Samples were collected from the anaerobic digester and aerobic oxidation basin of the Atlanta Municipal Sewage Plant in serum bottles which had been flushed previously with O₂-free N₂ gas; each serum bottle

was filled to the top with a sample. Within 3 h the serum bottles were brought back to a laboratory for enumeration and isolation of alkaliphilic thermophiles (15).

Isolation and culture conditions. The enrichment, isolation, and general culture methods used were basically the methods described previously (15). The initial isolation experiment was conducted at 50 and 60°C. YTG medium containing 0.5% (wt/vol) yeast extract, 1% (wt/vol) tryptone, and 0.3% (wt/vol) glucose in addition to the mineral base, reducing solution (15), and vitamin solution (15) was used for most of the experiments except for the tests of the growth requirement, substrate utilization (see below), dissimilatory sulfate reduction (15), and determination of the fermentation balance. Cultures were routinely incubated at pH 10.1 to 10.5 and 50°C. Growth requirements were determined at 50°C in a medium (pH 9.8) containing 50 mM Na₂CO₃, 1 mM KCl, 0.1% (wt/vol) NH₄Cl, 0.3% (wt/vol) glucose, 0, 0.01, or 0.02% (wt/vol) yeast extract, 5 ml of a trace element solution (15) per liter, 1 ml of a vitamin solution (15) per liter, and 1 ml of a reducing solution (15) per liter. Solid media contained 2.2% (wt/vol) agar (Difco Laboratories, Detroit, Mich.).

Light and electron microscopy. The morphological studies involving light microscopy and transmission electron microscopy were performed as described previously (15) by using a model PM-10AD phase-contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and a model JEM-100 CXII electron microscope (JEOL, Tokyo, Japan). The cells were negatively stained with 2% uranyl acetate by using the methods of Valentine et al. (26) and Beuscher et al. (2). Samples for ultrathin sectioning were prepared as previously described (7, 12, 25) by using uranyl acetate; lead citrate was used for poststaining. The Gram type (29) was determined by using the unequivocal lipopolysaccharide-polymyxin B assay (31).

pH and temperature ranges for growth. The temperature and pH ranges for growth were determined by using YT and YTG media as described previously (15). The various pH

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values were measured with a model 825-MP pH meter (Fisher Scientific) equipped with a combination pH electrode (Sensor, Staton, Calif.) and a Fisher Scientific temperature probe. The temperature range for growth was determined with a modified temperature gradient incubator (Scientific Industries, Inc., Bohemia, N.Y.) by using a temperature range of 25 to 70°C (variation, $\pm 0.5^\circ\text{C}$) and a shaking rate of 15 strokes per min.

Substrate utilization and fermentation products. Substrate utilization was determined by using a basic medium containing 0.1% (wt/vol) yeast extract, 50 mM Na_2CO_3 , 1 mM KCl, 2 mM Na_2PO_4 , 5 ml of a trace element solution per liter, and 1 ml of a reducing solution per liter, as previously described (15); this medium was supplemented with filter-sterilized prerduced test substrates (final concentration, 0.5% wt/vol). Because of the longer incubation times (up to 5 days), a pH of 9.6 was used to minimize caramelization of the medium ingredients at the elevated temperatures used. Increases in optical density at 600 nm (OD_{600}) of 0.1 U or more and changes in the pH compared with the control containing no added substrate were considered indications of positive results for substrate utilization. The maximum incubation time used was 7 days. Cultures which exhibited marginal growth responses (changes in OD_{600} of around 0.05 to 1.0) were subcultured to determine whether the cultures could be adapted to grow on the substrates in question.

The test for dissimilatory sulfate reduction was performed by using Hungate tubes containing medium which consisted of 0.3% (wt/vol) yeast extract, 0.5% (wt/vol) tryptone, 0.3% (wt/vol) glucose, 0.0025% cysteine hydrochloride, 0.1% Na_2SO_4 , and 0.01% FeSO_4 ; the pH of this medium was 10.1 or 8.5. The cultures were incubated at 50°C for up to 3 days, and two uninoculated tubes were used as controls.

The medium used to determine the fermentation balance contained 0.3% (wt/vol) yeast extract, 0.5% (wt/vol) tryptone, 50 mM Na_2CO_3 , 1 mM KCl, a trace mineral solution, a modified Wolfe vitamin solution, and a reducing solution as described above. The cultures were incubated at 50°C with and without 0.3% (wt/vol) glucose at pH 10.1 and 7.5. The contents of volatile and nonvolatile fatty acids (except acetate), alcohols, H_2 , and CO_2 were determined by gas chromatography. Acetate and glucose contents were determined by enzymatic assays as described previously (15).

Susceptibility to antibiotics. An exponentially growing culture was inoculated (3%, vol/vol) into fresh media containing filter-sterilized antibiotics (each at a concentration of 50 $\mu\text{g/ml}$). The cultures were incubated at 50°C and pH 7.5 or 10.1.

Effect of Na^+ and K^+ ions on growth and motility. An exponentially growing culture was inoculated into YTG media (pH 9.6 or 7.5) containing 0.25% (wt/vol) yeast extract, 0.25% (wt/vol) tryptone, 0.3% (wt/vol) glucose, and various amounts of NaCl and KCl. Before NaCl and KCl were added, the medium contained about 5 mM Na^+ and 4 mM K^+ .

DNA isolation and determination of G+C content. Purified DNA was prepared from cells in the early exponential growth phase by using the method of Ausubel et al. (1), which included CsCl gradient ultracentrifugation. The G+C content was determined after complete enzymatic digestion and high-performance liquid chromatography (HPLC) separation of the nucleosides as described by Whitman et al. (28) and Mesbah et al. (19).

16S rRNA sequence analysis. Total RNA was isolated from strain JW/YL23-2^T (T = type strain) by customary procedures (17, 33). We used the dideoxynucleotide chain termi-

nation method in which reverse transcriptase (21, 32) which was adapted for direct rRNA sequencing (14) was used. The synthesized strands were labeled by using [α - ^{35}S]thio-dATP (3). A standard set of primers (usually eight) specific for (eu)bacterial 16S rRNAs (14, 27, 32) was used routinely. All sequences were >90% complete. The sequences were aligned by using a sequence editor, ae2 (C. R. Woese, University of Illinois), with a representative collection of (eu)bacterial 16S rRNAs (20, 32). Corrected pairwise distances (expressed as the estimated number of changes per 100 nucleotides) were computed from percentages of similarity by using the Jukes-Cantor correction (11) as modified by G. J. Olsen (20) to accommodate the actual nucleotide ratios. Dendograms were constructed from evolutionary distance matrices by using the algorithm of De Soete (6).

Cell wall peptidoglycan analysis. Cell walls were prepared and peptidoglycan structure was determined by the methods described by Schleifer and Kandler (22). Amino acids and peptides were separated and characterized by thin-layer chromatography on cellulose sheets.

Nucleotide sequence accession number. The 16S rRNA nucleotide sequence of strain JW/YL23-2^T has been deposited in the GenBank data base under accession number L11304.

RESULTS AND DISCUSSION

Isolation. Slurries of sludge samples taken from either the aeration (oxidation) basin or the anaerobic digester of the sewage plant in Atlanta, Ga., were inoculated (5%, vol/vol) into fresh YTG medium (pH 10.5) and incubated at 50°C for 3 days. The cultures usually grew to a high density (OD_{600} , 0.6 to 1.0 U) overnight (about 6 to 9 h). The cultures were then serially diluted, and the two highest dilutions (10^{-5} and 10^{-6}) were used to inoculate YTG agar roll tubes (pH 10.1 to 10.5), which were incubated at 50°C for 1 to 3 days. Colonies having diameters of 0.3 to 0.5 mm appeared overnight (9 to 12 h). At least two different types of colonies were identified (*C. thermoalcaliphilum*-like and *C. paradoxum*-like; approximate ratio, 1:10). Single colonies were subsequently picked, immediately inoculated into fresh YTG medium, and characterized with respect to morphology and pH and temperature characteristics. Some colonies were white and circular and resembled *C. paradoxum* colonies (e.g., JW/YL15 colonies) (15). Cultures derived from two other colonies that had a yellow tint, isolates YL23-2 and YL24-1, were very similar. Isolate YL23-2 was chosen as the test strain used for further characterization. The purity of the strain JW/YL23-2 culture was ensured by three additional rounds of isolation of single colonies from agar shake roll tubes in which we used different variations of media (with and without glucose; different yeast extract and peptone contents; pH 7.8 and 10.5). The resulting culture was designated strain JW/YL23-2^T.

16S rRNA sequence analysis. The 16S rRNA sequence of strain JW/YL23-2^T was analyzed by examining its alignment as determined by the sequence editor ae2 with a representative collection of (eu)bacterial 16S rRNAs and by calculating the corrected pairwise (evolutionary) distances (expressed as estimated number of changes per 100 nucleotides) (Table 1). On the basis of evolutionary distances, strain JW/YL23-2^T belongs to the diverse genus *Clostridium* in the *Clostridium-Bacillus* subphylum. This strain exhibited a 2% evolutionary distance from the most closely related species, *C. paradoxum* (Fig. 1 and Table 1). The next most closely related species were *Clostridium sordellii*, *Eubacterium*

TABLE 1. Evolutionary distances for *C. thermoalcaliphilum* and other members of the clostridial subphylum^a

Organism	Evolutionary distance (%) to:																
	<i>Clostridium thermoaceticum</i>	<i>Clostridium pasteurianum</i>	<i>Clostridium butyricum</i>	<i>Clostridium perfringens</i>	<i>Clostridium oroticum</i>	<i>Clostridium aminovalericum</i>	<i>Clostridium purinolyticum</i>	<i>Clostridium lituseburense</i>	<i>Eubacterium tenue</i>	<i>Clostridium sordellii</i>	<i>Clostridium thermoalcaliphilum</i>	<i>Clostridium paradoxum</i>	<i>Clostridium manganotii</i>	<i>Clostridium sticklandii</i>	<i>Clostridium barkeri</i>	<i>Clostridium quercicolum</i>	<i>Megasphaera elsdenii</i>
<i>Clostridium pasteurianum</i>	17.9																
<i>Clostridium butyricum</i>	19.5	8.0															
<i>Clostridium perfringens</i>	19.3	9.4	7.3														
<i>Clostridium oroticum</i>	19.7	18.3	17.5	16.5													
<i>Clostridium aminovalericum</i>	19.3	18.0	17.9	17.3	8.3												
<i>Clostridium purinolyticum</i>	16.5	14.3	15.2	15.5	17.3	15.5											
<i>Clostridium lituseburense</i>	18.6	18.0	17.1	15.4	16.6	15.4	13.5										
<i>Eubacterium tenue</i>	17.7	16.9	17.5	14.4	15.7	15.7	13.0	3.9									
<i>Clostridium sordellii</i>	17.7	16.7	17.2	14.5	15.0	14.8	12.8	3.6	1.1								
<i>Clostridium thermoalcaliphilum</i>	16.2	17.3	17.9	14.5	16.5	17.2	13.2	9.6	7.5	7.8							
<i>Clostridium paradoxum</i>	15.6	17.2	18.0	15.0	17.4	17.7	13.3	9.5	7.8	8.0	1.9						
<i>Clostridium manganotii</i>	18.4	18.1	17.1	14.7	16.6	16.4	15.3	7.1	6.3	6.3	10.6	11.5					
<i>Clostridium sticklandii</i>	18.6	17.5	18.3	16.0	18.0	17.3	15.8	12.5	10.7	10.8	10.7	11.2	13.5				
<i>Clostridium barkeri</i>	18.3	17.4	19.2	17.6	20.4	19.4	14.8	18.7	17.8	18.0	18.8	19.1	18.1	19.7			
<i>Clostridium quercicolum</i>	16.4	21.1	22.8	22.8	22.8	21.7	18.4	21.1	21.1	21.1	21.0	21.2	20.8	23.0	21.3		
<i>Megasphaera elsdenii</i>	17.5	22.1	23.6	23.9	22.7	22.2	20.1	22.6	23.4	23.2	23.9	23.3	25.4	22.5	24.0	13.3	
<i>Bacillus subtilis</i>	15.9	16.0	18.0	17.5	20.1	19.5	16.7	18.8	18.0	18.1	17.4	17.4	18.9	19.6	17.3	18.0	21.1

^a Only those positions at which bases have been determined for all sequences in the set of organisms examined were used in the analysis. Approximately 1,290 positions were used in the analysis. *Bacillus subtilis* was used as an outgroup.

FIG. 1. Phylogenetic tree for *C. thermoalcaliphilum* and related organisms based on the results of a 16S rRNA sequence analysis (see Materials and Methods). Bar = 10 inferred changes per 100 bases. Abbreviations: C., *Clostridium*; E., *Eubacterium*; M., *Megasphaera*; B., *Bacillus*.

tenue, *Clostridium lituseburense*, and *Clostridium manganotii* (evolutionary distances, 7.8 to 10.6%). All of these organisms are substantially different from the type species of the genus *Clostridium*, *Clostridium butyricum*, and the species closely related to it, and which constitute the genus *Clostridium* sensu stricto. On the basis of distinct differences (see below) from *C. paradoxum* in 16S rRNA sequence, DNA G+C content, temperature range for growth, absence of spores, cell wall composition, and envelope structure, we concluded that strain JW/YL23-2^T is not a member of the species *C. paradoxum* or any other previously validly described species. Thus, until the genus *Clostridium* is redefined, strain JW/YL23-2^T is tentatively placed in a new species in this genus as *C. thermoalcaliphilum*.

***Clostridium thermoalcaliphilum* sp. nov.** *Clostridium thermoalcaliphilum* (ther. mo. al. cal. i. phil' um. Gr. adj. *thermo*, hot; M.L. n. *alcali*, from the arabic *al-qili*, the ashes of the saltwort plant; M.L. adj. *philum*, from Gr. adj. *philos*, dear, loving; M.L. adj. *thermoalcaliphilum*, referring to the organism's optimal growth under alkaline growth conditions at elevated temperatures). The following description is based on the properties of strain JW/YL23-2^T. The morphological, pH, and temperature characteristics of isolate YL24-1 are the same as those described below for strain JW/YL23-2^T.

Colony and cell morphology. Surface colonies are circular and white with a yellowish tint. Colonies in agar are lens shaped. Cells from liquid cultures in the exponential growth phase or prepared from 1- or 2-day-old colonies are highly motile rods that are 0.8 to 1.2 μ m in diameter and 3 to 12 μ m long (Fig. 2A). Cells in early- and mid-stationary-phase cultures are significantly longer (12 to 20 μ m) (Fig. 2B). Like *C. paradoxum*, the organism tends to develop pseudofila-

FIG. 2. Micrographs of *C. thermoalcaliphilum*. (A and B) Light micrographs. (A) Cells in the early exponential growth phase. (B) Cells in the stationary growth phase. Note the cells with swollen structures (arrowheads). (C through F) Electron micrographs. (C) Cell negatively stained with 2% uranyl acetate, showing peritrichous flagella. (D) Ultrathin section of a cell with a swollen body (arrow). (E and F) Ultrathin sections showing the fine structure of cell walls. The cultures were incubated at pH 10.1 and 50°C.

FIG. 3. (A and B) Effect of pH on growth as determined by maximum OD₆₀₀ (A) and doubling time (t_d) (B). (C) Effect of pH on motility. The cultures were incubated at 48°C in the presence (○) and in the absence (●) of 0.3% (wt/vol) glucose.

ments when it is grown at pH values of 10.9 to 11.0 (15). Swollen sporangium-like structures occur during growth at pH 10.1 to 11.0 (Fig. 2B, arrowheads, and Fig. 2D, arrow).

Cell wall, Gram-staining reaction, and Gram type. Transmission electron micrographs of ultrathin sections (Fig. 2D through F) show that strain JW/YL23-2^T has a cell envelope structure different from that of *C. paradoxum* (15), especially at the cell division junction, which seems to be sheathed by an envelope structure (Fig. 2E and F). Furthermore, peptidoglycan structure analysis shows that the cell wall type of strain JW/YL23-2^T is A4β (L-Orn-D-Asp), while the cell wall type of *C. paradoxum* is A1τ (*m*-diaminopimelic acid direct). The Gram-staining reaction is negative irregardless of the growth stage; however, since no lipopolysaccharide-polymyxin B complex is detected, the organism is gram type positive (29, 31).

Sporulation tests. No endospores are formed after growth under various culture conditions, including after growth in agar roll tubes containing agar-solidified nutrient-deficient xylan media (at alkaline and neutral pH values) incubated for up to 1 week. Heat-treated cultures at all growth stages do not grow when they are incubated at 48 to 50°C and pH 10.0 after the heat treatment. Cultures in the exponential or

FIG. 4. Effect of temperature (T) on growth (○) and motility (●). The pH of the cultures was 10.1. t_d , doubling time.

stationary growth phase left at room temperature for more than 1 week frequently cannot be subcultured, indicating that spores were not formed. The swollen structures observed (Fig. 2D), which somewhat resemble prespores in their overall shape, contain only irregular refractile substances and apparently are not heat-stable spores. As the cultures become older, these structures eventually swell further and burst. Transmission electron micrographs of ultrathin sections of the swollen structures do not indicate that *C. thermoalcalophilum* develops membrane structures that are associated with the formation of prespores (Fig. 2D) like those observed with *C. paradoxum* (15). These results indicate that this organism does not form viable spores under the conditions tested, although on the basis of the 16S rRNA sequence analysis results, it is closely related to the spore-forming organism *C. paradoxum* (see below).

Motility and flagellation. The organism is motile from the early exponential growth phase through the stationary growth phase. The cells have 2 to 12 peritrichous flagella (Fig. 2C). The flagella are 10 to 15 μm long and 90 to 95 nm in diameter and have a wavelength of 2.5 to 3.0 μm.

Motility depends on the pH of the growth medium (Fig. 3) and the incubation temperature (Fig. 4). The organism is motile at pH values ranging from 7.0 to 10.6; the optimum pH for motility is around 9.8, which coincides with the optimum pH for growth (Fig. 3A and B). Less than 5% of the cells are motile in the absence of glucose; however, in the presence of 0.3% (wt/vol) glucose at pH 9.8 the percentage of motile cells is 65% (Fig. 3C). When a culture that is growing exponentially at 50°C is used as the inoculum, motility occurs at temperatures ranging from 30 to 53°C (optimum temperature, 42 to 52°C) (Fig. 4).

Growth properties. (i) **Growth requirements.** The organism is obligately anaerobic. No growth occurs when an exponentially growing culture is inoculated into aerobically prepared YTG medium, nutrient broth, or agar-solidified media. In the absence of yeast extract and tryptone, no growth occurs when an exponentially growing culture is inoculated into basic mineral media (pH 9.8) containing 0.1% (wt/vol) NH₄Cl as the nitrogen source and 0.3% (wt/vol) glucose as the carbon and energy source. However, some growth (OD₆₀₀, 0.15 U) does occur when 0.02% (wt/vol) yeast extract is added, indicating that yeast extract is required for growth. Tryptone but not with peptone can be used as a substitute for yeast extract.

(ii) **Temperature and pH ranges for growth.** The temperature range for growth at pH 10.1 is 27 to 57.5°C; the optimum

FIG. 5. Effect of Na⁺ concentration on growth (maximum OD₆₀₀) in the presence of 25 mM K⁺ (○) and 100 mM K⁺ (●).

temperature for growth is between 48 and 51°C (Fig. 4). The pH range for growth is 7.0 to 11.0 at 50°C; the optimum pH for growth is between 9.5 and 10.0 (Fig. 3). The same results are obtained in the presence (YTG medium) and absence (YT medium) of glucose (Fig. 3). Under optimal conditions (pH 10.1, 50°C, YTG medium), the shortest doubling time that has been observed with strain JW/YL23-2^T is 19 min. This value is similar to the observed doubling time for *C. paradoxum* and relatively small compared with the reported doubling times of most other glycolytic thermophiles (30).

(iii) **Effect of NaCl and KCl on growth.** The Na⁺ concentration range for growth is 5 mM to 1.25 M (Fig. 5). The maximum tolerated Na⁺ concentration (1.25 M) is much higher than the maximum tolerated Na⁺ concentration for *C. paradoxum* (15) (about 0.75 M NaCl). The range of Na⁺ concentrations at which growth occurs in the presence of 25 mM K⁺ (5 mM to 1.25 M) is a little wider than the range of Na⁺ concentrations at which growth occurs in the presence of 100 mM K⁺ (20 to 990 mM) (Fig. 5). A higher maximum OD₆₀₀ occurs in the presence of 25 mM K⁺ than in the presence of 100 mM K⁺ for all of the Na⁺ concentrations

that have been tested. Near the maximum tolerated NaCl concentration (1.25 M in the presence of 25 mM K⁺ and of 990 mM in the presence of 100 mM K⁺), the morphology of the cells changes, leading to the formation of L-form cells after 24 h of incubation at 50°C. At NaCl concentrations higher than the maxima, the cells begin to lyse within 2 h. At the lower NaCl concentrations tested (less than 5 mM in the presence of 25 mM K⁺ and less than 20 mM in the presence of 100 mM K⁺), the cells do not grow, and the normal morphology deteriorates with prolonged incubation (after 6 h), as the concentration of NaCl decreases.

Substrate utilization and fermentation products. In the presence of 0.1% (wt/vol) yeast extract (pH 9.6), *C. thermoautotrophicum* utilizes glucose, fructose, sucrose, maltose, cellobiose, and Casamino Acids. It does not utilize galactose, lactose, xylose, ribose, mannose, rhamnose, raffinose, salicin, sorbitol, mannitol, glycerol, lactate, formate, methanol, xylan, cellulose, starch, pectin, and H₂-CO₂. The fermentation products formed at pH 10.5 and 50°C from the utilization of yeast extract and 2.3 μmol of glucose per ml include 16.1 μmol of acetate per ml, 3.7 μmol of H₂ per ml, 1 μmol of isovalerate per ml, 0.7 μmol of lactate per ml, trace amounts (<0.5 μmol/ml) of succinate and malate, and one unidentified product with a retention time of 14.7 to 14.8 min, but no significant amounts of bicarbonate and carbonate. Differences in product formation occur among the four conditions which we tested (Table 2). The unidentified product formed in the presence and absence of glucose has the same chromatographic properties as the product formed under similar conditions by *C. paradoxum* (15).

Biochemical properties. (i) Dissimilatory sulfate reduction. No sulfate reduction activity occurs in the presence of glucose, acetate, and lactate, thus making it unlikely that isolate JW/YL23-2^T is a sulfate-reducing organism.

(ii) **Fatty acid analysis.** The main fatty acids are (in order of decreasing amount formed) i15:0, a15:0, i13:0, 16:0, i17:0, 14:0, and 18:0. The ester-linked phospholipid fatty acid methyl ester profile for cells grown at pH 10.0 to 10.5 is quite similar to the profile of *C. paradoxum* grown at pH 7.5 (15) but is distinct from the profile of *C. paradoxum* cultured at pH 10.5. It is interesting that *C. paradoxum* has a phospho-

TABLE 2. Fermentation product analysis for *C. thermoautotrophicum*^a

Substrate or product	Amt (μmol/ml) used (substrate) or formed (products) ^b			
	Culture 1 (pH 10.5, no glucose)	Culture 2 (pH 10.5, 0.3% glucose)	Culture 3 (pH 7.5, no glucose)	Culture 4 (pH 7.5, 0.3% glucose)
Glucose substrate	0.05	2.3	0.02	1.0
Products				
Acetate	4.6	16.1	4.8	3.7
H ₂	0.9	3.7	1.4	1.0
CO ₂	0	0	2.4	1.7
Isovalerate	0	1.0	1.0	0.8
Lactate	Tr	0.7	0	0
Malate	Tr	Tr	0	Tr
Pentanol	Tr	Tr	Tr	Tr
Succinate	Tr	Tr	0	0
Unidentified ^c	++	+	+	++

^a Cultures of strain JW/YL23-2^T were grown at 50°C for 10 h in YT medium containing 0.3% yeast extract and 0.5% tryptone or YTG medium containing 0.3% yeast extract, 0.5% tryptone, and 0.3% glucose at pH 10.5 or 7.5. A culture in the early exponential growth phase was used as the inoculum (5%, vol/vol). Tr, trace (<0.5 μmol/ml).

^b After 10 h the final pH values of cultures 1 through 4 were 10.4, 9.9, 7.0, and 6.8, respectively. The biomasses of cultures 1 through 4 were 0.08, 0.26, 0.06, and 0.07 mg (dry weight)/ml.

^c A relatively large peak in the gas chromatogram with a retention time of 14.7 to 14.8 min. +, peak area between 1,000 and 2,000; ++, peak area between 9,000 and 15,000.

lipid fatty acid methyl ester profile similar to that of non-spore-forming strain JW/YL23-2^T at pH 7.0 to 8.0, pH values at which *C. paradoxum* does not form spores (15). However, at the present time no further conclusion can be drawn from this information.

(iii) **API An-Ident tests.** An-Ident tests (API Analytab Products, Plainview, N.Y.) result in positive responses for indole acetate, α -glucosidase, arginine aminopeptidase, and arginine utilization. Negative responses occur in tests for *N*-acetyl-glucosaminidase, β -glucosidase, α -fucosidase, phosphatase, α -galactosidase, β -galactosidase, leucine aminopeptidase, proline aminopeptidase, pyroglutamic acid arylamidase, tyrosine aminopeptidase, alanine aminopeptidase, glycine aminopeptidase, arabinosidase, indole production, and catalase. Strain JW/YL23-2^T differs from *C. paradoxum* (Table 3) in the results for four tests (the indole production, α -arabinosidase, arginine aminopeptidase, and catalase tests).

(iv) **Susceptibility to antibiotics.** We tested the effect of antibiotics at 50°C. At pH 7.5 and 10.1, *C. thermoalcaliphilum* is susceptible to 50 μ g of penicillin G per ml, 50 μ g of ampicillin per ml, 50 μ g of metroimidazole per ml, 50 μ g of gentamicin per ml, and 50 μ g of rifampin per ml. In agreement with 16S rRNA data, this suggests that this organism is a (eu)bacterial species. For a more meaningful assessment, a detailed study of the thermostability of the antibiotics used at pH 10 and 50°C needs to be done.

(v) **DNA G+C content.** The DNA G+C content, as determined by complete digestion of DNA with P1 nuclease and alkaline phosphatase followed by HPLC analysis (15, 19, 28), is 32 mol%.

Maintenance. Since so far no endospores have been observed and cultures stored at room temperature frequently become nonviable within 1 week, the cultures need to be preserved in liquid nitrogen or as 50% glycerol-containing culture suspensions at -18 or -75°C. Such cultures are viable for at least 1 year.

Type strain. Strain JW/YL23-2, which was isolated from a sewage plant in Atlanta, Ga., is the type strain of *C. thermoalcaliphilum* sp. nov. and has been deposited in the Deutsche Sammlung von Mikroorganismen as strain DSM 7309; its G+C content is 32 mol%.

Differentiation of *C. thermoalcaliphilum* from *C. paradoxum* and other alkaliphilic obligate anaerobes. *C. thermoalcaliphilum* is a thermotolerant (temperature range for growth, 27 to 57.5°C) (30) facultative alkaliphile since it grows at pH values below 8.0. Its pH range for growth is very similar to that of *C. paradoxum* (15). These two organisms have other properties in common; they are both Gram-type positive but Gram stain negative (29, 31), obligately anaerobic, motile by means of peritricous flagella, and sulfate reduction negative, have short doubling times under alkaline culture conditions, and produce mainly acetate and an unknown fermentation product (Tables 2 and 3). They differ from each other, however, by 2% in both DNA G+C content and evolutionary distance derived from 16S rRNA sequence analysis. They have different cell wall compositions and different cell wall morphologies (see above). So far, *C. thermoalcaliphilum* has not been observed to form viable heat-stable endospores, while *C. paradoxum* sporulates readily under most experimental conditions (15). *C. thermoalcaliphilum* is thermotolerant, while *C. paradoxum* is a thermophile with a temperature range for growth of 30 to 63°C (15). These organisms are the only two obligately anaerobic, alkaliphilic (by the stringent definition of Krulwich and Guffanti [13, 15]) species that have been described

TABLE 3. Major differences between *C. thermoalcaliphilum* and *C. paradoxum*^a

Species	Cell wall type	Spore formation	DNA G+C content (mol%)	Temp range for growth (°C)	Optimum temp (°C)	API An-Ident tests ^b			
						Indole production	α -Arabinosidase	Arginine aminopeptidase	Catalase
<i>C. thermoalcaliphilum</i>	A48 (L-Orn-D-Asp), with sheath covering the junction between dividing cells	No sporulation	32	27-57.5	48-51	-	-	+	-
<i>C. paradoxum</i>	A17 (m-diaminopimelic acid direct), with no sheath-like structure	High sporulation rate	30	30-63	56	w	+	-	w

^a In addition, as determined by a 16S rRNA analysis, the evolutionary distance between *C. thermoalcaliphilum* and *C. paradoxum* is about 2%.

^b -, negative; +, positive; w, weakly positive.

so far. Both *C. thermoalcaliphilum* and *C. paradoxum* are distinctly different from the previously described obligately anaerobic, mesophilic, spore-forming alkaliphile isolated by Souza et al. (24), which grew at pH 8.0 to 11.3 and 27 to 30°C. This isolate was not further characterized, and its description was not validly published; in addition, as indicated above, this organism was not available to us for comparison.

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REFERENCES

- Ausubel, F. M., et al. 1989. Current protocols in molecular biology, p. 2.4.1-2.4.5. Wiley Interscience, New York.
- Beuscher, N., F. Mayer, and G. Gottschalk. 1974. Citrate lyase from *Rhodospseudomonas gelatinosa*: purification, electron microscopy and subunit structure. *Arch. Microbiol.* **100**:307-328.
- Biggin, M. D., T. J. Gibson, and G. F. Hing. 1983. Buffer gradient gels and ³⁵S label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA* **80**:3963-3965.
- Blotevogel, K., U. Fischer, M. Mocha, and S. Jannsen. 1985. *Methanobacterium thermoalcaliphilum* spec. nov., a new moderately alkaliphilic and thermophilic autotrophic methanogen. *Arch. Microbiol.* **142**:211-217.
- Boone, D. R., S. Worakit, I. M. Mathrani, and R. A. Mah. 1986. Alkaliphilic methanogens from high-pH lake sediments. *Syst. Appl. Microbiol.* **7**:230-234.
- De Soete, G. 1983. A least square algorithm for fitting additive trees to proximity data. *Psychometrika* **48**:621-626.
- Frasca, J. M., and V. R. Parks. 1965. A routine technique for double-staining ultrathin sections using uranyl and lead salts. *J. Cell Biol.* **25**:157-161.
- Grant, W. D., and K. Horikoshi. 1988. Alkaliphiles. *FEMS Symp.* **49**:346-366.
- Grant, W. D., W. E. Mwatha, and B. E. Jones. 1990. Alkaliphiles: ecology, diversity and applications. *FEMS Microbiol. Rev.* **75**:255-270.
- Horikoshi, K. 1990. Microorganisms in alkaline environments. Kodansha, Tokyo.
- Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21-132. In H. N. Munro (ed.), *Mammalian protein metabolism*. Academic Press, New York.
- Kellenberger, E., A. Ryter, and J. Sechaud. 1958. Electron microscope study of DNA-containing plasma. II. Vegetative and mature phage DNA as compared with normal bacterial nucleosides in different physiological states. *J. Biophys. Biochem. Cytol.* **4**:671-678.
- Krulwich, T. A., and A. A. Guffanti. 1989. Alkalophilic bacteria. *Annu. Rev. Microbiol.* **43**:435-463.
- Lane, D. J., B. Pace, G. J. Olsen, and D. A. Stahl. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. USA* **82**:6955-6959.
- Li, Y., L. Mandelco, and J. Wiegel. 1993. Isolation and characterization of a moderately thermophilic alkaliphile, *Clostridium paradoxum* sp. nov. *Int. J. Syst. Bacteriol.* **43**:450-460.
- Liu, Y., D. R. Boone, and C. Choy. 1990. *Methanohalophilus oregonense* sp. nov., a methylotrophic methanogen from an alkaline, saline aquifer. *Int. J. Syst. Bacteriol.* **40**:111-116.
- Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**:208-218.
- Mathrani, I. M., D. R. Boone, R. A. Mah, G. E. Fox, and P. P. Lau. 1988. *Methanohalophilus zhilinae* sp. nov., an alkaliphilic, halophilic, methylotrophic methanogen. *Int. J. Syst. Bacteriol.* **38**:139-142.
- Mesbah, M., U. Premachandran, and W. B. Whitman. 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int. J. Syst. Bacteriol.* **39**:159-167.
- Olsen, G. J., R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W. M. Kuan, T. J. Macke, and C. R. Woese. 1992. The ribosomal database project. *Nucleic Acids Res.* **20**(Suppl.):2199-2200.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
- Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**:407-477.
- Shiba, H., H. Yamamoto, and K. Horikoshi. 1989. Isolation of strictly anaerobic halophiles from the aerobic surface sediments of hypersaline environments in California and Nevada. *FEMS Microbiol. Lett.* **57**:191-196.
- Souza, K. A., P. H. Deal, H. M. Mack, and C. E. Turnbill. 1974. Growth and reproduction of microorganisms under extremely alkaline conditions. *Appl. Microbiol.* **28**:1066-1068.
- Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**:31-43.
- Valentine, R. C., B. M. Shapiro, and E. R. Stadtman. 1968. Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *E. coli*. *Biochemistry* **7**:2143-2152.
- Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. van Etten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J. Bacteriol.* **171**:6455-6467.
- Whitman, W. B., S. Sohn, D. S. Caras, and U. Premachandran. 1986. Isolation and characterization of 22 mesophilic methanococci. *Syst. Appl. Microbiol.* **7**:235-240.
- Wiegel, J. 1981. Distinction between the gram reaction and the Gram type of bacteria. *Int. J. Syst. Bacteriol.* **31**:88.
- Wiegel, J. 1992. The obligate anaerobic thermophilic bacteria, p. 105-184. In J. K. Kristjansson (ed.), *Thermophilic bacteria*. CRC Press, Boca Raton, Fla.
- Wiegel, J., and L. Quandt. 1982. Determination of the Gram type using the reaction between polymyxin B and lipopolysaccharides of the outer cell wall of whole bacteria. *J. Gen. Microbiol.* **128**:2261-2270.
- Woese, C. R., R. Gutell, R. Gupta, and H. F. Noller. 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiol. Rev.* **47**:621-669.
- Woese, C. R., M. Sogin, D. A. Stahl, B. J. Lewis, and L. Bonen. 1976. A comparison of the 16S ribosomal RNAs from mesophilic and thermophilic bacilli. *J. Mol. Evol.* **7**:197-213.
- Worakit, S., D. R. Boone, R. A. Mah, M. Abdel-Samie, and M. M. El-Halwagi. 1986. *Methanobacterium alcaliphilum* sp. nov., an H₂-utilizing methanogen that grows at high pH values. *Int. J. Syst. Bacteriol.* **36**:380-382.