Isolation and Characterization of a Moderately Thermophilic Anaerobic Alkaliphile, *Clostridium paradoxum* sp. nov.

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Alkaliphilic, moderately thermophilic anaerobic bacteria able to grow above pH 10.5 and 55°C were isolated from various sewage plants in the United States. The strains were motile with two to six peritrichous flagella and formed round to slightly oval terminal spores in terminally distended and slightly enlarged cells. Sporulated cells remained motile. The pH range for growth was between 7.0 and 11.1, with an optimum of around 10.1. At pH 10.1 the temperature range for growth was between 30 and 63°C, with an optimum of 56°C. The shortest observed doubling time (glucose) was around 16 min at 56°C and pH 10.1. No dissimilatory sulfate reduction was detected. The organism utilized glucose, fructose, sucrose, maltose, and pyruvate but required yeast extract or tryptone for growth. Optimal NaCl concentrations for growth were between 50 and 200 mM. The guanine-plus-cytosine content was 30.0 ± 0.10 mol%. On the basis of unique properties and 16S rRNA analysis, the strains are placed in a new species, *Clostridium paradoxum*, referring to the unusual retainment of motility by sporulated cells. Strain JW-YL-7 (DSM 7308) is designated as the type strain.

In recent years, alkaliphiles have attracted considerable attention in both fundamental and applied research (10, 11, 26). One of the most stringent definitions for alkaliphiles is given by Krulwich and Guffanti (16), requiring an organism to have an optimum pH for growth (pH_{opt}) of 10.0 and above to be counted as an alkaliphile. Whereas facultative alkaliphiles can grow in the neutral pH range, the obligate alkaliphiles cannot grow below pH 8.5. Alkaliphilic bacteria have been isolated from a variety of environments, representing a broad spectrum of taxonomic groups, with the most extensively studied being aerobic mesophilic strains belonging to the genus *Bacillus* (11, 15, 16). Khraptsova (14) reported alkalitolerant thermophilic aerobes with an optimum growth temperature (T_{opt}) of around 50°C (pH_{opt} , 8.0). According to Horikoshi (11), the highest reported growth temperature for an alkaliphilic aerobe, *Bacillus* sp. strain 221 (ATCC 2152), an alkaline protease producer, is 57°C. However, little is known about alkaliphilic anaerobes, and even less is known about alkaliphilic thermophilic anaerobes. Among the archaea, one thermophilic (*Methanobacterium thermoalkalophilum*: maximum growth temperature [T_{max}], <73°C; T_{opt} , 58 to 62°C; pH_{opt} , 7.5 to 8.5; maximum pH for growth [pH_{max}], <10) (5), one thermotolerant (*Methanohalophilus zhilinae*: T_{max} , 55°C, T_{opt} , 45°C; pH_{max} , <10.5; pH_{opt} , 9.2) (6), and several mesophilic methanogens, capable of growth at pH values above pH 9.5, were isolated during the last decade (18, 21, 38). In 1974, Souza et al. (28), reported the isolation of a mesophilic sporeforming anaerobe that grew between pH 8.0 and 11.3, but no subsequent characterization or other studies of the organism were reported. Recently, Shiba et al. (27) reported the isolation of four mesophilic strains of strictly anaerobic alkalitolerant halophiles which grew between pH 6.5 and 10.0, and Nimura et al. (23) described a facultative anaerobe, *Amphibacillus xylanus*, which grows between pH 8.0 and 10.0. To our knowledge, there has been no other report on the isolation and characterization of alkaliphilic anaerobic (eu-)bacteria since the report of Souza et al. (28).

In this report, we describe the isolation and characterization of an alkaliphilic thermophilic anaerobe capable of growth at pH 11.0 and at temperatures above 60°C. We propose to place the described strains in the new species *Clostridium paradoxum* sp. nov.

MATERIALS AND METHODS

Isolation and culture conditions. One to two milliliters of liquid and 5 to 10 g of solid anaerobic and aerobic samples from anaerobic waste digestors and aeration pools (Table 1) were inoculated into 9 ml of prereduced medium in Hungate tubes (borosilicate glass, 16 by 125 mm; Bellco Glass, Inc., Vineland, N.J.) or 50 ml of prereduced medium in 150-ml serum bottles (Wheaton 400 borosilicate glass; Fisher Scientific Co., Pittsburgh, Pa.), respectively. The enrichment cultures were incubated at 50 to 60°C (higher temperatures lead to rapid deterioration of the complex growth medium). The medium was prepared by the Hungate technique (19), using an oxygen-free nitrogen atmosphere. The yeast extract-tryptone (YT) medium contained per liter 994 ml of mineral base (consisting of 50 mM Na_2CO_3 , 2 mM Na_2HPO_4 , and 1 mM KCl), 5 ml of trace element solution (9), 1 ml of vitamin solution (modified Wolfe solution [9]), 0.125 g of Na_2S , 0.125 g of cysteine acid, 1 mg of resazurin (as a redox indicator), 5 g (or as indicated) of yeast extract, and 10 g (or as indicated) of tryptone. For the glucose-amended medium (YTG medium), prereduced sterile glucose was added separately to a final concentration of 0.3% (wt/vol) or as indicated. After the media were autoclaved, the pH was adjusted to 10.5 or as indicated using anaerobic sterile 2 N sodium hydroxide or potassium hydroxide. The amounts of yeast extract and tryptone were varied for specific purposes. The YTG medium for the enumeration contained the above-mentioned mineral base supplemented with 0.5% yeast extract, 1% tryptone, and 0.3% glucose, pH 10.1 to 10.3 (around the pH_{opt} for this organism). If not stated differently, the purified cultures were routinely grown in the same YTG medium at pH 10.1 to 10.5. The pH of the media was measured with an Accumet pH Meter (model 825 MP; Fisher Scientific) equipped with a combination pH electrode

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TABLE 1. Enumeration of *C. paradoxum* and other alkaliphilic anaerobic thermophiles

Location, temperature, and pH of samples collected	Alkaliphilic bacteria/ ml of sample ^a	<i>C. paradoxum</i> -like strains isolated
Anaerobic digester, Athens, Ga.; 38°C, pH 7.8	500–1,000	JW/YL-A1, JW/YL-A3, JW/YL-A11
Aeration pool, Athens, Ga.; ambient, pH 6.9–7.5	500–1,000	JW-YL-1, JW-YL-2, JW-YL-4, JW-YL-7 ^T , JW-YL-8, JW-YL-12
Anaerobic digester, Atlanta, Ga.; 38–42°C, pH 7.9	1,000–10,000	JW/YL-L1, JW/YL-L5
Aeration pool, Atlanta, Ga.; ambient, pH 7.4	100–1,000	JW/YL-L15, JW/YL-L21
Anaerobic digester, Beaverton, Oreg.; 37°C, pH 6.4–7.0	100–1,000	JW/YL-01, JW/YL-05, JW/YL-09
Anaerobic digester, Miami, Fla.; 25–30°C, pH 7.1	100	JW/YL-F1, JW/YL-F6, JW/YL-F10
Anaerobic digester, Gulf Breeze, Fla.; ambient, pH 7.5–7.0	50–100	JW/YL-F11, JW/YL-F12
Anaerobic digester, Norman, Okla.; 35°C, pH 7.0–7.4	100–500	JW/YL-Ok1, JW/YL-Ok2, JW/YL-OK5
Digester effluent, San Francisco, Calif.; ambient, pH 7.0	10–50	JW/YL-SF4, JW/YL-SF5
Anaerobic digester, San Francisco, Calif.; 37°C, pH 7.3	100–1,000	JW/YL-SF6, JW/YL-SF7, JW/YL-SF8

^a The most probable number determination was done at pH 10.1 and 60°C using YTG medium; additional strains were isolated growing at 55 but not at 60°C.

(Sensor, Stanton, Calif.) and a temperature probe (catalog no. 13-620-16; Fisher Scientific) for temperature corrections. The isolation of pure cultures from liquid enrichment cultures (50 to 60°C) was done by repeated subculturing at 50°C and pH 10.8, the highest pH still yielding moderately fast growth, followed by four or more rounds of isolation of single colonies in agar shake-roll tubes (19), using YTG or YTP medium (pH 10.5 to 11.0, 50°C) amended with 2% agar (Difco Laboratories, Detroit, Mich.). This included isolation of colonies from heat-treated (5 min, 100°C) inocula containing sporulated cells. YTP medium contained 0.3% yeast extract, 0.3% tryptone, and 0.2% peptone plus the same mineral base. Growth was determined by measuring the increase in optical density at 600 nm (OD₆₀₀) with a Spectronic 21 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.).

To further ensure the purity of the type culture, strain JW-YL-7^T was repeatedly cultured (at 50°C) in four separate lines, an alkaline line at pH 10.8 to 11.0 and a neutral one at pH 7.4 to 7.6, each with or without glucose; these established cultures were then subjected twice to separate serial dilutions (final dilutions, 1:1) into liquid medium. The highest dilutions grown up under each set of conditions were heat treated (100°C for 2 min) and then inoculated into agar-solidified medium. Cultures incubated at pH 7.5 (with or without glucose) were not subjected to heat treatment because of their failure to form heat-stable spores at neutral pH (see below). The four lines of cultures finally obtained (alkaline line with and without glucose and neutral line with and without glucose) were each grown under all four conditions and compared with respect to morphology, sporulation, and doubling times.

Light and electron microscopy. A phase-contrast microscope (Model PM-10AD; Olympus Optical Co., Ltd., Tokyo, Japan) was used for routine examination and for obtaining photomicrographs of the organism. Electron microscopy (negative staining and ultrathin section) was performed with a JEM-100 CXII Electron Microscope (JEOL, Tokyo, Japan). Negative staining of cells was done with 2% uranyl acetate according to Valentine et al. (30) and Beuscher et al. (3). The preparation of samples for ultrathin sectioning was done as described previously (8, 13, 29), using uranyl acetate and lead citrate for poststaining. The Gram type was determined by using the unequivocal lipopolysaccharide-polymyxin B assay as described previously (35).

pH and temperature ranges. The pH range for growth was determined by growing the bacteria at 50°C in Hungate tubes. The YT medium (used in order to avoid interference

through caramelization of sugars at the extreme pH values) was kept within ± 0.1 pH unit of the initial pH by frequent adjustment with anaerobic sterile 2 N NaOH. Growth was determined by monitoring the increase of the OD₆₀₀ against three incubated blank control tubes. Cultures were incubated for up to 10 days to determine no-growth boundaries. The temperature range for growth was determined in Hungate tubes containing 5 ml of YT or YTG at pH 10.1 in a temperature gradient incubator (Scientific Industries, Inc., Bohemia, N.Y.) set from 20 to 71°C with a shaking speed of 15 rpm.

Substrate utilization. To a base medium (50 mM Na₂CO₃, 1 mM KCl, 2 mM Na₂HPO₄, 0.1% [wt/vol] yeast extract, 5 ml of trace element solution per liter, 1 ml of vitamin solution per liter, and reducing agents as described above [pH 9.6]), sterile anaerobic test substrates (0.5% [wt/vol] final concentration) were added. The suboptimal pH was chosen to minimize possible alterations (Lobry deBruyn reaction) of various carbohydrates at the alkaline pH values. Tubes were inoculated with 2% (vol/vol) of a culture exponentially growing at 50°C. Increase in optical density and pH changes were monitored. Two controls (inoculated base medium containing 0.2% [wt/vol] yeast extract and no added carbon source) were set up for each test strain. Reported OD₆₀₀ values were corrected for growth in these controls. Substrate utilization was assumed when an increase in OD₆₀₀ of 0.1 or above was recorded. Assays were performed in duplicate. Cultures showing marginal growth (OD₆₀₀ between 0.07 and 0.14) were subcultured for confirmation of growth on this substrate; the maximal incubation time was 10 days.

Fermentation products. H₂, CO₂, and CH₄ were determined on a gas chromatograph (Varian, Walnut Creek, Calif.) equipped with a Porapak Q (80/100 mesh) column (Supelco, Inc., Bellefonte, Pa.), a flame ionization detector (for CH₄ determinations), and a thermal conductivity detector (for CO₂ and H₂ determinations). Volatile fatty acids and alcohols, as well as nonvolatile fatty acids, were analyzed as described previously (34) by using a Hewlett-Packard 5880A Gas Chromatograph (Hewlett-Packard, Avondale, Pa.) equipped with a Chromosorb WAW 10% sp-100 glass column (Supelco) and a flame ionization detector. Glucose and acetate were determined by enzymatic assays (catalog no. 139106 and 148261, respectively; Boehringer GmbH, Mannheim, Germany).

Dissimilatory sulfate reduction. The dissimilatory sulfate reduction test was performed with 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%

TABLE 2. Evolutionary distances between *C. paradoxum* and selected members of the clostridial subphylum^a

Organism	Evolutionary distance																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. <i>C. thermosaccharolyticum</i>																		
2. <i>C. thermoaceticum</i>	13.0																	
3. <i>C. pasteurianum</i>	18.7	17.6																
4. <i>C. butyricum</i>	19.6	19.3	7.8															
5. <i>C. perfringens</i>	18.0	19.2	9.4	7.4														
6. <i>C. oroticum</i>	21.5	19.3	17.8	17.2	16.2													
7. <i>C. aminovalericum</i>	21.0	19.0	18.0	17.8	17.0	8.1												
8. <i>C. purinolyticum</i>	18.1	16.2	14.4	15.2	15.3	16.9	15.3											
9. <i>C. lituseburens</i>	20.6	18.2	17.9	16.8	15.2	15.9	15.2	13.5										
10. <i>E. tenue</i>	19.8	17.4	16.8	17.4	14.4	15.2	15.6	13.0	3.8									
11. <i>C. sordellii</i>	20.0	17.4	16.7	17.1	14.6	14.7	14.9	12.9	3.7	1.2								
12. <i>C. paradoxum</i>	17.5	15.4	17.1	18.1	15.2	17.1	17.7	13.3	9.5	7.7	7.9							
13. <i>C. manganotii</i>	21.0	18.4	18.1	17.1	14.9	16.2	16.2	15.3	6.9	6.3	6.3	11.5						
14. <i>C. sticklandii</i>	20.5	18.3	17.6	18.3	16.0	17.7	17.3	15.8	12.4	10.7	10.7	11.0	13.4					
15. <i>C. barkeri</i>	18.7	17.9	17.1	19.0	17.5	20.2	19.2	14.6	18.3	17.5	17.8	18.9	18.1	19.4				
16. <i>C. quercicolum</i>	19.3	16.1	20.8	22.5	22.6	22.3	21.2	18.1	20.8	20.7	20.8	20.9	20.6	22.7	20.6			
17. <i>M. elsdenii</i>	19.7	17.2	22.1	23.4	23.9	22.3	21.9	20.1	22.5	23.3	23.0	23.0	25.1	22.3	23.4	12.9		
18. <i>B. subtilis</i>	19.2	15.4	15.8	17.8	17.4	19.5	19.3	16.5	18.5	17.8	18.0	17.1	18.7	19.4	16.8	17.5	21.1	

^a Only those positions having a known base in all sequences in the set of organisms were used in the analysis. Approximately 1,290 positions were used in the analysis. C., *Clostridium*; B., *Bacillus*; E., *Eubacterium*; M., *Megasphaera*. *B. subtilis* served as an outgroup.

cysteine · HCl, 0.1% Na₂SO₄, and 0.01% FeSO₄, pH 10.1. The cultures were incubated at 50°C for up to 5 days. Two uninoculated tubes were used as controls.

Susceptibility to antibiotics. Susceptibility to antibiotics was determined by transferring (3%, vol/vol) an exponentially growing culture into fresh YTG medium containing 5 to 100 µg of filter-sterilized O₂-free antibiotics per ml and incubating at 50°C and pH 7.4 and 10.1. Attempts to subculture the incubated cultures in medium without antibiotics were used to test for a bacteriostatic or bacteriocidal effect of the antibiotics.

Metal content of the medium and sewage sludge samples. The concentrations of Na⁺, K⁺, and other metal ions in the sediment samples from the Athens sewage plant as well as in the employed media were determined with a plasma emission spectroscope (Jarrell-Ash 965 ICP; Thermo Jarrell Ash Corporation, Franklin, Mass.).

Determination of mole percent G+C. DNA was isolated according to Ausubel et al. (2), using CsCl gradient ultracentrifugation. The guanine plus cytosine content (mole percent G+C) was determined after enzymatic digestion and high-performance liquid chromatography (HPLC) separation of the nucleosides as described by Whitman et al. (32) and Mesbah et al. (22).

Effect of Na⁺ and K⁺ on growth, motility, and sporulation. The effect of sodium ion on growth, motility, and sporulation was investigated by inoculating 3% (vol/vol) of an exponentially growing culture into a YTG medium (pH 9.6 and pH 7.5 without Na carbonate but containing various amounts of NaCl, KCl, NaOH, or KOH and 0.25% yeast extract, 0.25% tryptone, and 0.3% glucose).

16S rRNA isolation, sequencing method, and data analysis. The 16S rRNA was isolated from the two strains JW-YL-7^T and JW/YL-L15 by customary procedures (20, 37). The dideoxynucleotide chain termination method (4, 25) adapted for direct rRNA sequencing using reverse transcriptase (17) was employed. Synthesized strands were labeled by the inclusion of [α -³⁵S]thio-dATP (4). A standard set of primers (usually eight) specific for (eu)bacterial 16S rRNAs (4, 17, 31) was routinely used. All sequences were >90% complete. The sequences were aligned in the sequence editor ae2

(C. R. Woese, University of Illinois) against a representative collection of (eu)bacterial 16S rRNAs (24, 36). Corrected pairwise distances (expressed as estimated changes per 100 nucleotides) were computed from percent similarities by using the Jukes and Cantor correction (12) as modified by G. J. Olsen (described in reference 31) to accommodate the actual nucleotide ratios. Dendograms were constructed from evolutionary distance matrices by using the algorithm of De Soete (7).

FIG. 1. Phylogenetic tree for *C. paradoxum* and related organisms based on 16S rRNA sequences (see Materials and Methods and Table 2). The bar represents 10 inferred changes per 100 nucleotides. C., *Clostridium*; B., *Bacillus*; M., *Megasphaera*. *Bacillus subtilis* has been used to root the tree.

FIG. 2. Light micrographs of *C. paradoxum* in early (A) and late (B) exponential, stationary (C), and death or free-spore (D) phases.

Nucleotide sequence accession number. The GenBank accession number for the 16S rRNA sequence of the type strain is L06838.

RESULTS AND DISCUSSION

Enumeration of alkaliphilic thermophilic anaerobes. At pH 10.2 and 60°C, most probable number counts (Table 1) of alkaliphilic thermophilic anaerobes in sewage sludge samples taken from various sewage plants (anaerobic digestors and aeration pools) in the United States ranged from about 50 to 10,000 bacteria per ml of wet sample (3 to 7% solid content). The tested effluent samples (sewage plants in San Francisco, Calif., and Athens, Ga.; see discussions below), had significantly lower counts than the corresponding anaerobic digester samples. At the Athens, Ga., site, two samples of water and sediment were taken from the discharge channel of the sewage plant; two further sediment samples were taken from the bank of the Oconee River at 0.1 and 2 to 3 km downstream from the sewage plant. Furthermore, samples were taken 0.2 km upstream from the plant. The enrichment

and enumeration (most probable number) of these samples, however, failed to yield any organism similar to the organism isolated from the aeration pools and anaerobic digesters. Although others (10, 11, 15, and 16 and authors cited therein) and, recently, we have isolated many (over 150) alkalitolerant strains from various samples, including from hot springs, forest soils, agricultural soils, saline lakes, and sediments from rivers and freshwater lakes (unpublished data), no facultative or obligate alkaliphiles (using the definition of Krulwich and Guffanti [16]) were obtained from these samples.

Isolation of alkaliphilic thermophilic strains. About 29 strains of anaerobic bacteria, able to grow above pH 10.5 at 60°C, were isolated as described in Materials and Methods from samples obtained from sewage plants in the United States (Table 1). The isolates proved to be quite similar to one another, especially the strains from Athens and San Francisco, which were very much alike in morphology, sporulation, and motility of sporulated cells. Similar strains were isolated from the anaerobic digester sludge of the plant in Athens and from the aeration pool, which periodically received return sludge from the clarifier and anaerobic

FIG. 3. Light (A) and electron (B) micrographs of *C. paradoxum* L-form cells. The arrow indicates an L-form cell within the phase-contrast microphotograph. (C) Light microscopy (phase contrast) of filamentous cells grown at extreme alkaline pH (10.9 to 11.1).

digestor. To our knowledge, this is the first isolation of truly alkaliphilic anaerobic thermophilic bacteria.

Because of the observed morphological variability and the biphasic pH curves (see below) strain JW-YL-7^T was established in four different culture lines (representing four different growth conditions as described in Materials and Methods) to verify the uniformity of this strain. Consequently, each line was grown at 50°C under those four sets of conditions, i.e., in the presence and absence of glucose at pH 10.8 to 11.0 (alkaline lines) and 7.4 to 7.6 (neutral lines). A comparison of properties (including morphology, motility, extent of sporulation, and doubling times) revealed no differences among the four lines when subcultured under the same conditions. We concluded that the culture JW-YL-7^T is an axenic strain. The biphasic pH curve was observed with all the strains tested (JW-YL-1, JW-YL-2, JW-YL-4, JW-YL-8, JW-YL-12, and JW/YL-L15). Thus, on the basis of these results and the extended tests done with the type strain, JW-YL-7, we conclude that the biphasic pH curve is a general feature for *C. paradoxum* and that, with respect to the other tested strains, the biphasic pH dependency curves do not indicate that the cultures were impure.

16S rRNA analysis. The 16S rRNA sequence analysis of strains JW-YL-7^T and JW/YL-L15 placed them into the *Clostridium-Bacillus* subphylum (Table 2; Fig. 1). The two

strains were indistinguishable at the 16S rRNA level. *Clostridium sordellii* and *Eubacterium tenue* are the closest related species (Table 2; Fig. 1). Thus, the two strains are clearly separated from the cluster of species around the type species *Clostridium butyricum*, which are regarded as clostridia sensu stricto. The new strains are also separated from the other two anaerobic thermophiles for which sequence analyses were available, *Clostridium thermoaceticum* and *Clostridium thermosaccharolyticum*. Consequently, on the basis of the results of the 16S rRNA analysis, the unique pH and temperature range for growth, and the motility of sporulated cells, we concluded that the isolated strains do not belong to a validly published species. Thus, the strains were placed tentatively into a new clostridial species until the genus *Clostridium* is redefined, the relationship to other thermophiles has been elucidated on the 16S rRNA level, and an International Committee of Systematic Bacteriology subcommittee has established rules for reclassification of the presently described *Clostridium* species.

Description of *Clostridium paradoxum* sp. nov. The following characterization of the new clostridial species is based on the properties of the type strain, JW-YL-7. Physiological and morphological properties were corroborated with the strains JW-YL-1, JW-YL-2, JW-YL-4, JW-YL-8, JW-YL-12, and JW/YL-L15, isolated from the sewage plant in Athens and

FIG. 4. Electron micrographs of ultrathin sections of dividing cells. (A to F) Early stage; (G to H) later stage; (I) completion of division. Note the frequently observed formation of excess membranes at the site of cell division.

Atlanta, Ga. (Table 1). On the basis of the ability to grow at the high pH values of 10.5 and above, the morphology of the vegetative and sporulated cells, and the motility of the sporulated cells, the additional strains listed in Table 1 are assumed to belong to the same species. However, no numerical taxonomy has been done to further verify this assumption.

Clostridium paradoxum sp. nov.; para.do.xum. Gr. *para* (akin to Gr. *pro*, before, ahead) plus *doxos* (fr. Gr. *dokein*, to seem, to think, opinion); Gr. *paradoxon*, fr. neut. of *paradoxos*, contrary to expectation; M.L. *paradoxum*, referring to the unusual property of sporulated cells to be highly motile.

Colony morphology. In agar shake-roll tubes containing YTP medium without glucose at pH 10.0 to 10.5 and 50°C, the agar surface colonies were white, translucent, and circular to slightly irregular. Colonies in the agar were lens shaped and creamy white. No pigmentation was observed when cells were grown in either liquid or on solidified YTP

and YTG media. After 3 to 5 days, colonies usually started to turn transparent as the cells began to form autoplasts (L-form) and to lyse.

Morphology of vegetative cells. Exponential cells, grown in liquid or agar-solidified medium, were rods of 0.7 to 1.1 μm in diameter and 2 to 4.5 μm in length. Chains of two and more cells were seen in the early exponential phase. When grown at the optimal pH of 10.1 and at 50°C, the cells from the exponential growth phase were regular to slightly bent rods (Fig. 2A). The morphology became more pleiomorphic in the stationary growth phase (within 6 to 20 h), with slightly deformed, swollen or curved cells (Fig. 2B and C). Occasionally, L-form types of cells were seen, mainly during prolonged incubation (1 to 2 days or longer) at either elevated temperatures (55 to 60°C) or low pH values (around pH 7.1, 50°C) close to the minimum pH for growth (Fig. 3A). Some of these cells still possessed flagella (Fig. 3B) and stayed motile. At or near the pH_{max} (10.9 to 11.1) at 50°C or around 60 to 63°C at pH 10.1, the organism frequently

FIG. 5. Effect of initial pH on growth (maximal OD₆₀₀) (A), motility (B), and sporulation (C). ○, 3 h; ●, 6 h; □, 25 h; ■, 72 h. YTG medium was used; incubation was at 50°C.

developed into filamentous rods (Fig. 3C), with a length of up to 50 µm, without visual septation or changes in diameter. The electron micrographs revealed a variation in cell division. Some stages suggest a pinching mode (Fig. 4A to C) while others indicate more of a septation mechanism (Fig. 4D to H). Frequently, dividing cells showed the formation of excess membrane structures (Fig. 4C to H), indicating that the cell division process might be inhibited. On the other hand, at this time, it cannot be excluded that this is an artifact caused by the fixation process for electron microscopy.

Gram stain reaction and Gram type. The Gram stain reaction was negative, regardless of the growth phase; however, the test for the formation of the lipopolysaccharide-polymyxin B complex gave no indication of the presence of lipopolysaccharide (35). Thus, the organism is regarded as a Gram-type-positive organism (31). This is also indicated by the fine structure of the cell wall, although atypically thin for a Gram-type-positive bacterium, as revealed in the photomicrographs of ultrathin sections (Fig. 4). This is also in agreement with the data from the 16S rRNA sequence analysis, indicating that the organism belongs to the Gram-type-positive *Clostridium* and *Bacillus* subphylum (Fig. 1).

Sporulation. Generally, high rates of sporulation (up to 90%) were observed in both liquid and solidified agar YTG media, leading to the formation of swollen sporangia of the "drumstick" type (Fig. 2B and C). Spores were round and 1

to 1.1 µm in diameter. In the absence of glucose, sporulation frequency decreased to 10 to 20%. During growth on glucose, the majority of the cells sporulated after 24 to 48 h of incubation. The sporulation was pH dependent (Fig. 5). At pH 7.0 to 7.5, no sporulation was seen with the vegetative cells, which were wider in diameter (1 to 1.1 µm) than at pH 10.1 (0.7 to 1.0 µm). During growth at pH 10.9 to 11.0, the vegetative cells developed into a pseudofilamentous form and the frequency of sporulation decreased from 85 to 95% (at pH 10.1) to 1 to 5%. The formation of spores was usually observed during the late exponential or early stationary phase; the ratio of spores and vegetative cells remained relatively constant during the stationary phase until the inception of death phase, when the cells began to lyse and the released spores accumulated into large clumps (Fig. 2D). The clumps of spores were seen in both shaken and unshaken cultures. Frequently, the formation of structures resembling prespores was seen on the distal end of the sporulated mother cell (Fig. 2B and C), with no visible septation (Fig. 6A and B). The electron micrograph of ultrathin sections revealed the typical endospore structure for the fully developed spore (Fig. 6C). Sporulation was also dependent on concentrations of Na⁺ and K⁺ added to the medium (Fig. 7), exhibiting a very sharp optimum (50 to 150 mM NaCl) in the absence of added KCl. No sporulation occurred below 10 mM NaCl regardless of whether 25 or 100 mM added KCl was present.

Motility and flagellation. The motility of the cells was pH dependent (Fig. 5) and was also influenced by the concentrations of sodium and potassium ions present in the culture medium (Fig. 7). At pH 10.1, exponentially growing cells were motile, as were most of the cells in early or mid-stationary growth phase. Surprisingly, sporulated cells were highly motile, with swimming in the direction of either end (i.e., containing the spore or not) of the cell. The sporulated cells exhibited longer phases of smooth swimming than the vegetative cells, which appeared to tumble most of the time. Electron micrographs revealed two to six peritrichous flagella per cell of up to 12 to 15 µm in length and 90 to 92 nm in diameter (Fig. 8), with a flagellum wave length of 2.0 to 2.2 µm (Fig. 8A). The subunits are helically packed along the filament axis of the flagellum with an angle of less than 60° formed between the axis of the longitudinal rows of subunits and the axis tangential to the left or right oblique rows of subunits (Fig. 8B), representing the type II flagellum as defined by Aragno et al. (1).

Growth properties. (i) **Effects of pH and temperature on growth.** The pH range for growth was 7.3 to 11.0 (tested at 50°C), with an optimum between pH 9.8 and 10.3 (Fig. 9A). By the stringent definition of Krulwich and Guffanti (16), the isolates are facultative alkaliphiles. In contrast to aerobic alkaliphiles which lower or raise the pH in the medium to their optimal pH, as shown by Ueyama and Horikoshi (Fig. 2.3, reference 11), none of the isolated anaerobic strains tested exhibited this phenomenon; because of the formation of acidic fermentation products, the pH became more acidic. The average change in pH ranged in the absence of glucose from 0.1 to 0.5 pH unit and 0.4 to 1.0 pH unit in the presence of 0.3% added glucose.

The marginal temperature data for growth in YTG medium (pH 10.1) were as follows: T_{max}, >62°C and <63.5°C; T_{opt}, 50 to 58°C; T_{min}, <32°C and >30°C (incubation for up to 5 days; longer incubation times render the medium unsuitable). No significant differences were observed in the growth temperature optimum and range when the cells were grown in the presence and absence of glucose (Fig. 9B). With YT

FIG. 6. Electron micrographs of ultrathin sections of sporulated cells (A and B) and endospore (C). Note the formation of the highly swollen cell end (A and B, arrow), which resembled the formation of an additional prespore-like structure on the distal end of the sporulated cell.

medium (0.5% yeast extract, 0.3% tryptone), the doubling time at the pH_{opt} of 10.1 and T_{opt} of 55°C was 16 min in the presence of 0.5% glucose and 22 min in the absence of glucose. This is the shortest doubling time observed for a strict anaerobic bacterium, regardless of the extreme environment. We speculate that this short doubling time could be one of the reasons that this alkali-thermophile can be found in relatively high numbers in mesophilic sewage sludge with pH values usually around neutral but, which, especially in pockets of the anaerobic sludge vessel, can undergo shifts in pH and temperature for short periods of time and thus render the environment temporarily favorable for these organisms to grow. However, the monitoring data we received from several of the sewage plants did not show any values above 38°C and pH 7.6.

(ii) Effects of Na^+ and K^+ on growth. At pH 9.6, the

FIG. 7. Effect of sodium ion concentration on growth (maximal OD_{600}) (A and D), motility (B and E), and sporulation (C and F) in the absence (A, B, and C) and presence (D, E, and F) of added potassium ions. Closed symbols represent incubation at pH 7.5, and all open symbols represent incubation at pH 9.6. Concentrations of added KCl: $\cdots\circ\cdots$, 25 mM, 2 h; $\cdots\square\cdots$, 100 mM, 2 h; $-\circ-$, 25 mM, 4 and 10 h; $-\square-$, 100 mM, 4 and 10 h. The tests were done with cultures incubated at 50°C in YTG medium containing 0.25% yeast extract, 0.25% tryptone, 0.3% glucose, and in addition sodium and potassium salts at sodium and potassium ion concentrations of between 4 and 6 and 3 and 5 mM, respectively. Similar results were obtained in the absence of glucose (data not shown).

FIG. 8. Electron micrograph of a negatively stained cell with two flagella (A) and high magnification of a segment of a flagellum exhibiting the helical arrangement of the flagellin subunits (see text for further explanation) (B).

optimal sodium concentration for growth (maximal OD_{600}) was between 50 and 100 mM, while at pH 7.5 the range was extended to from 50 to 500 mM (Fig. 7). The addition of 25 or 100 mM KCl increased the stimulation by NaCl and shifted the inhibitory effect of sodium ions to higher concentrations; e.g., in the presence of added potassium ion, the optimum concentration for growth was between 150 and 300 mM NaCl instead of 50 to 100 mM NaCl without added potassium. At both alkaline and neutral pH values and in the presence and absence of added potassium ions, 1 M NaCl inhibited growth completely. Potassium had less of a stimulatory effect on growth than on motility and sporulation. In

the presence of 25 and 100 mM added KCl, the optimum and inhibitory concentrations of added NaCl were shifted to higher concentrations (Fig. 7). Furthermore, in the presence of 25 to 100 mM potassium, the extent of the sodium-induced effect was dependent on the culture age; e.g., at the early exponential growth phase (2 h) a higher percentage of cells were motile compared with the percentages in the late exponential growth phase and the transition phase (4 and 10 h, respectively). Furthermore, at the early exponential growth phase the optimal NaCl concentration was around 50 to 100 mM NaCl. Similar data were obtained in the absence of glucose (data not shown).

FIG. 9. Effects of pH (A) and temperature (B) on the growth rate of *C. paradoxum* JW-YL-7. The pH profile was obtained with YTP medium at 50°C, and the temperature profile was obtained with YT (●) and YTG (○) media at pH 10.1.

TABLE 3. Fermentation product analysis^a

pH ^b	Glucose added	Glucose used (μmol/ml)	Amt (μmol/ml) of product						Unidentified ^c	Biomass (mg [dry wt] ml ⁻¹)
			Acetate	CO ₂	H ₂	Isovalerate	Succinate	Butyrate		
10.5	0%	0	3.3	4.3	2.3	Trace	Trace	Trace	+	0.26
	0.3%	6.2	10.5	5.6	4.8	0.8	0.7	Trace	+	0.5
7.5	0%	0	2.1	6.7	2.5	1.5	Trace	Trace	+	0.2
	0.3%	3.2	2.8	8.1	2.3	0.7	Trace	Trace	+	0.26

^a Cultures were grown at 50°C for 7 h in YT (0.3% yeast extract, 0.5% tryptone) and YTG (YT + 0.3% glucose) at both pH 10.5 and 7.5. A culture in an early exponential growth phase was used for a 5% (vol/vol) inoculum. Trace = <0.5 μmol/ml.

^b Final pHs of the cultures were 10.4, 10.0, 7.0, and 6.8, respectively.

^c Response peak in the gas chromatogram with a retention time of 14.84 min and a peak area of 5,000 or above.

The aeration pool samples and the anaerobic digester samples from the Athens Sewage Plant had similar sodium concentrations (3 to 3.5 mM) but differed slightly in the potassium concentrations (0.5 versus 1.2 mM). These data suggest that *C. paradoxum* and alike strains live in the sewage sludge at suboptimal Na⁺ and K⁺ concentrations.

(iii) **Substrates and fermentation products.** No growth was observed under aerobic conditions, i.e., when aerobically prepared YTG, YTP, and nutrient broth (Difco) media, either liquid or solidified with 2% agar and with a pH of 10.1, were used. The organism requires prerduced (i.e., resazurin-colorless) medium containing either yeast extract or tryptone, which cannot be replaced by peptone. The addition of 0.1 to 0.5% (wt/vol) peptone to media containing 0.3% (wt/vol) yeast extract or tryptone had no beneficial effect on growth. Substrates which were utilized in the presence of 0.1% (wt/vol) yeast extract included glucose, fructose, sucrose, maltose, and pyruvate. In addition, some strains were able to use lactose (JW-YL-4 and JW-YL-7^T) and cellobiose and xylitol (JW-YL-2 and JW-YL-7^T). The substrates not utilized were galactose, xylose, ribose, L-(+)-arabinose, mannose, mannitol, rhamnose, raffinose, salicin, sorbitol, malonate, glycerol, lactate, formate, pectin, starch, cellulose, ethanol, methanol, H₂-CO₂, and Casamino Acids. Moreover, the addition of 0.5% (wt/vol) D-mannose, methanol, and ethanol inhibited growth, compared with growth in unamended yeast extract-containing base medium. With or without glucose, at pH 7.5 and 10.5, acetate, CO₂, and H₂ were the major products; however, one product could not be identified (Table 3).

Biochemical properties. (i) **Dissimilatory sulfate reduction.** No dissimilatory sulfate reduction was detected, thus excluding the organism from the sporeforming, dissimilatory sulfate-reducing genus *Desulfotomaculum*.

(ii) **Fatty acid analysis.** PLFAME (ester-linked phospholipid fatty acid methyl ester) profiles were determined (Table 4). Under alkaline growth conditions (pH 10.1 to 10.5), the patterns of PLFAME profiles of strains JW-YL-7^T, JW-YL-8, and JW/YL-L15-1 were similar but were different from the one obtained for an asporogenous strain, JW/YL-23-2 ("*C. thermophilum*"; unpublished data), isolated from sewage sludge from the Atlanta Sewage Plant. However, when JW-YL-7^T was grown at pH 7.4 to 7.6, at which it does not form spores, its PLFAME pattern was similar to that of the Atlanta strain. However, further experiments, isolation of further strains, and data for other thermophiles and alkaliphiles are required before further conclusions can be drawn.

(iii) **An-Ident tests.** In An-Ident tests (API Analytab Products, Plainview, N.Y.), strong positive results were obtained for the following reactions: α-glucosidase, indoxylacetate hydrolysis, arginine aminopeptidase, and arginine

utilization. Weak positive reactions were found for indole production and catalase production. The other reactions (*N*-acetylglucosaminidase, L-arabinosidase, β-glucosidase, α-fucosidase, α-galactosidase, β-galactosidase, leucine aminopeptidase, proline aminopeptidase, pyroglutamic acid arylamidase, tyrosine aminopeptidase, alanine aminopeptidase, histidine aminopeptidase, phenylalanine aminopeptidase, and glycine aminopeptidase) were negative. The same results were obtained for incubations at either pH 8.5 or 10.5 and at 37 or 50°C.

(iv) **Sensitivity to antibiotics.** Strain JW-YL-7^T was tested in YTG medium at both pH 7.4 and 10.1. Growth was inhibited by 25 μg of chloramphenicol, erythromycin, tetracycline, monesin, gramicidin S, and lasalocid per ml and 50 μg of gentamicin per ml. The inhibition spectrum is in agreement with that for strains belonging to the (eu)bacteria.

(v) **G+C content (mole percent).** The guanine-plus-cytosine content of the genomic DNA was 30.0 ± 0.1 mol% for the six tested strains, JW-YL-1, -2, -4, -7^T, -8, and -12, as determined by complete P1 nuclease and alkaline phosphatase digestion and HPLC analysis of the nucleosides (22, 32).

Type strain. Strain JW-YL-7, isolated from the sewage plant in Athens, Ga., is designated as the type strain of *Clostridium paradoxum* and is deposited with the German Culture Collection (DSM 7308). The G+C content of the

TABLE 4. Phospholipid fatty acid pattern (PLFAME analysis)

PLFAME	%			
	JW-YL-7 ^T		JW-YL-8	JW/YL-L15
	pH 7.5	pH 10.5	(pH 10.5)	(pH 10.5)
i13:0	4.42	0.81	0.91	1.03
a13:0	0.70	0.20	0.22	0.24
13:0	0.11	0.05	0.05	0.05
i14:0	0.56	0.22	0.20	0.21
14:0	5.96	2.07	2.77	2.44
i15:0	65.04	60.63	60.85	64.98
a15:0	7.24	7.77	7.92	7.84
15:0	1.21	1.30	1.75	1.44
i16:0	0.99	1.23	1.23	1.05
16:1w7c	0.19	0.10	0.12	0.09
16:0	6.08	7.23	8.21	5.92
i17:0	4.27	12.79	11.02	10.67
a17:0	0.79	3.05	2.80	2.29
17:0	0.13	0.26	0.23	0.17
18:2w6	0.06	0.05	0.04	0.08
i18:0	0.44	0.30	0.29	0.27
18:1w7c	0.27	0.17	0.14	0.14
18:1w7t	0.12	0.14	0.18	0.06
18:0	1.42	1.64	1.05	1.01
Total pmol/mg	49,263	68,255	66,440	63,747

type strain is 30 mol%. The properties are as described above for the species.

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