Isolation and Characterization of a Cellulolytic Microorganism, *Cellulomonas fermentans* sp. nov.

C. BAGNARA,* R. TOCI, C. GAUDIN, AND J. P. BELAICH

Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, 13277 Marseille Cedex 9, and
Université de Provence, Marseille, France*

A new mesophilic, cellulolytic microorganism was isolated from a municipal dumping ground. The isolation of this bacterium was performed under anaerobic conditions, but its growth was similar under both anaerobic and aerobic conditions. This gram-positive, nonmotile, coryneform rod, with a high (75.8 mol%) guanine-plus-cytosine content in deoxyribonucleic acid (DNA), can grow on numerous carbon sources. The fermentation products are acetic acid, ethanol, formic acid, succinic acid, CO₂, and, occasionally, lactic acid. Nucleic acid hybridization tests between the DNA of the isolate and the DNA of *Cellulomonas uda* ATCC 21399 showed some partial (37 to 40%) relatedness. This information, in addition to other data, suggests that this organism belongs to the genus *Cellulomonas*, and it is proposed here as a new species, *Cellulomonas fermentans* sp. nov. The type strain is M (DSM 3133).

The utilization of solid municipal waste is of interest from both economic and pollution abatement points of view. Among the methods proposed so far for waste disposal purposes are composting and anaerobic digestion (26, 35). In municipal dumping grounds, spontaneous methane production has been found to occur. Because municipal refuse contains as much as 50% paper (1) in addition to other cellulosic substrates, cellulose mineralization is probably an important part of the decomposition process.

A cellulolytic bacterium was isolated under anaerobic conditions from an ecosystem of this type. This paper reports how it was identified and characterized.

MATERIALS AND METHODS

Media and culture conditions. The basal medium used had the following composition: K₂HPO₄, 2.21 g; KH₂PO₄, 1.50 g; (NH₄)₂SO₄, 1.30 g; MgCl₂ · 6H₂O, 0.10 g; CaCl₂ · 2H₂O, 0.02 g; FeSO₄ solution (0.5% [wt/vol]), 0.25 ml; yeast extract, 5 g; and distilled water to bring the final volume to 1 liter. The pH was adjusted to 7.4 with 8 N NaOH.

For isolation purposes, the basal medium was supplemented with 4.0% (wt/vol) ball-milled MN300 cellulose and 2.0% agar and used by the Hungate roll-tube technique (19).

For anaerobic cultures in liquid medium, after autoclaving (30 min, 120°C), 10 ml of sterile cysteine-hydrochloride solution (5.0%) and 10 ml of sterile sodium bicarbonate solution (8.0%) per liter were added. The anaerobic culture technique used was that of Hungate (18) as modified by Bryant (7). Hungate anaerobic tubes were used for 10-ml cultures. For more voluminous cultures, we used special flasks (Schott) sealed with butyl rubber stoppers and screw caps.

When carbon sources were soluble carbohydrates, they were autoclaved separately (30 min, 110°C) and added just before inoculation.

For culture maintenance, the basal medium was supplemented with 0.5% MN300 cellulose and dispensed in 10-ml volumes into Hungate tubes under an O₂-free argon atmosphere.

All cultures were incubated at 30°C without agitation.

Isolation procedures. Strain M^T was obtained by enrich-

ment and isolation of bacteria from Montaubert (Essonne, France) municipal dumping ground samples taken at the depth of 23 m.

Enrichment culture was initiated by the inoculation of about 15 g of sample into a 250-ml culture flask which contained 100 ml of basal medium supplemented with 8% barley straw, under anaerobic conditions.

We ascertained that methane production occurred during the 5 weeks of incubation.

Serial dilutions in 25 mM phosphate buffer from sterile samples of this culture were then prepared and used to inoculate cellulose agar roll tubes. After 2 weeks, colonies developing cleared zones of cellulose digestion were individually transferred into liquid basal medium supplemented with 0.2% cellobiose. This procedure was repeated a few more times to ascertain the purity of the culture by colony-type homogeneity and microscopic examination.

Morphology. Living and stained cells were examined by light microscopy.

For electron microscopy, a 2-day-old culture grown on 0.2% cellobiose was used. After dilution of the culture in distilled water, a drop of the cell suspension was placed on a Formvar-coated copper grid, and the preparation was fixed and stained with a drop of 1% phosphotungstic acid solution.

For thin-section preparation, cells were fixed in glutaraldehyde and OsO₄ as described by Ryter and Jacob (24), dehydrated, and embedded in araldite. Thin sections were contrasted with uranyl acetate and lead citrate.

Growth measurements. Growth on soluble carbohydrate medium was measured by optical density (450 nm). For growth on cellulose, the pellet proteins were determined as described by Bensadoun and Weinstein (2), using bovine serum albumin as the standard.

Temperature and pH for optimal growth. Basal medium supplemented with 0.2% cellobiose was used to determine the optimal growth temperature. For each temperature tested, five tubes, each containing 10 ml of medium, were inoculated with 5% (vol/vol) of a 2-day-old culture grown on a similar medium at 30°C. Optical densities were read directly at 450 nm on a Pye Unicam spectrophotometer. The growth rate average was calculated for each temperature tested.

For pH studies, a modified basal medium supplemented with 0.2% cellobiose was used; phosphate buffer was re-

* Corresponding author.

placed by 200 mM MOPS buffer (morpholinepropanesulfonic acid)–5 mM KH_2PO_4 . Cultures (50 ml) with pH adjusted from 6 to 8 (by increments of 0.4 pH units) just before sterilization were inoculated with a 2-day-old culture grown in the same medium. Growth measurements were performed after sterile sampling on a Kontron spectrophotometer (model Uvikon 810). The pH was checked in each sample, and it did not decrease more than 0.1 unit from the initial pH. The growth rate average was calculated for each pH tested.

Physiological tests. (i) Nutritional requirements. To determine amino acid and vitamin requirements, yeast extract from the maintenance broth was extensively diluted by four successive transfers into yeast extract-free basal medium supplemented with 0.2% cellobiose (cellobiose minimal medium [CMM]) before inoculation. Volumes (100 ml) of the nine following media were inoculated: CMM, CMM plus biotin (0.2 mg/liter) plus thiamine (0.5 mg/liter), CMM plus vitamin cocktail (0.2 ml/liter), CMM plus vitamin-free Casamino Acids (0.2%), and CMM plus various yeast extract concentrations (0.01, 0.05, 0.1, 0.2, and 0.5%). The composition of the vitamin cocktail was *p*-aminobenzoic acid (0.025 g), biotin (0.01 g), nicotinamide (0.015 g), panthothenic acid (0.025 g), cyanocobalamin (0.01 g), and distilled water to obtain 100 ml. Yeast extract and Casamino Acids were autoclaved in the medium, and vitamins sterilized by filtration were then added aseptically.

(ii) Carbon source utilization. Basal medium with only 0.05% yeast extract was used to test for the utilization of numerous substrates as sole carbon source. These substrates were autoclaved separately and added to the sterile medium to give a final concentration of 0.2%. Control cultures were grown on the medium with no other carbon source, and each test was carried out in duplicate.

(iii) Biochemical tests. Catalase and nitrate reduction tests were conducted as described by Holding and Collee (17). For catalase production, a comparative test was performed on nutrient agar in a petri dish between strain M^T and *Cellulomonas uda* ATCC 21399 (catalase positive). Nitrate reduction was tested in basal medium containing 0.1% KNO_3 .

To test cytochrome oxidase, gelatinase, and β -galactosidase, Biomerieux (France) methods and reagents were used.

Ammonia production from peptone was tested as described by Stackebrandt and Kandler (29), and indole production was tested as described by Holdeman et al. (16).

(iv) Fermentation end-product analyses. Culture samples were centrifuged for 20 min at $12,000 \times g$. The supernatants obtained were used for determining alcohols and acids.

Ethanol, acetic acid, and L-(+)-lactic acid were determined spectrophotometrically with the appropriate enzyme kit (Boehringer GmbH, Mannheim, Federal Republic of Germany). Formic acid was determined by the colorimetric method of Lang and Lang (21).

Succinic acid and acetylmethylcarbinol were assayed with a Girdel gas chromatograph fitted with a Chromosorb 101 (80/100 mesh) column (1.5 m long by 6.4 mm in diameter). The chromatograph was fitted with a flame ionization detector, and the following conditions were selected for the analysis: carrier gas, 1.2 bar (1.2×10^5 Pa) of argon at the entrance to the column; injector and detector temperatures, 250°C.

For succinic acid evaluation, a 0.25-ml sample was methylated as described by Holdeman et al. (16), and 1 μl of the methylated sample was injected into the gas chromatograph. The column temperature was 210°C.

For acetylmethylcarbinol evaluation, a 0.25-ml sample was acidified with 25 μl of 12 N HCl, and 1 μl of acidified supernatant was injected into the gas chromatograph. The column temperature was 160°C.

Gases were analyzed by removing a sample (0.5 ml) from the headspace of a culture tube with a 1-ml syringe and injecting it into a Girdel chromatograph fitted with a thermoconductivity detector (100 mA) and silica gel and molecular sieve columns (both 3.2 mm in diameter and 1.5 m long). For both columns, the temperature was 80°C, and the carrier gas was argon at a pressure of 1.0 bar (1.0×10^5 Pa) at the entrance to the column. The injection and detector temperatures were 150°C.

Cell wall amino acid analysis. Fully hydrolyzed cell wall material was prepared from approximately 10 mg (dry weight) of cells by the hot trichloroacetic acid-trypsin rapid-screening method of Schleifer and Kandler (25). Chromatography of amino acids was performed with the two-dimensional thin-layer chromatography system described by Harper and Davis (15).

Cell wall carbohydrate analysis. The method described by Stanek and Roberts (32) was used for cell wall carbohydrate analysis.

DNA base composition and hybridization. (i) Preparation of DNA and analysis of DNA base composition. The method described by Stackebrandt and Kandler (29) was used for the preparation of the deoxyribonucleic acid (DNA). The melting temperature of the purified DNA was determined with a Perkin-Elmer spectrophotometer (model 551) by the method of Mandel and Marmur (23), and the guanosine-plus-cytosine (G+C) content was calculated. The accuracy of the method was determined with DNA of *Micrococcus lysodeikticus* (G+C, 72 mol%) and of *Escherichia coli* (G+C, 50 mol%) as standards.

Labeled DNA was extracted from cells which were cultivated in basal medium supplemented with 0.2% cellobiose (as for unlabeled DNA) and 1 mCi of [^3H]thymidine per 300 ml of medium. Cells were harvested at the end of the log phase and disrupted with glass beads (diameter, 0.45 to 0.50 mm) in a Braun breaking apparatus for 2 min at a breaking speed of 1,500 oscillations per min, and then the DNA was purified by the same method as that used for unlabeled DNA.

All DNA concentrations were determined by measuring absorbance at 260 nm.

The specific radioactivity of the ^3H -labeled DNA obtained ranged between 14×10^3 and 43×10^3 cpm/ μg of DNA.

(ii) Preparation of DNA filter. Denatured unlabeled DNA was prepared by the method of Birnstiel et al. (4), and DNA filters were made as described by Stackebrandt and Kandler (29). Nitrocellulose membrane filters (25-mm diameter; 0.22- μm pore size [type GSWP; Millipore Corp., Bedford, Mass.]) were used. The binding efficiency of the DNA to nitrocellulose filters ranged between 84 and 95% of the DNA input.

For the experiments, four small identical filters, each containing about 37 μg of DNA, were cut out of one filter.

(iii) Hybridization procedure. For fragmentation, solutions of labeled DNA were sonically treated (three times 15 s with 15-s intervals) with a Sonifier (model S110; Branson Sonic Power Co., Danbury, Conn.) at 50% intensity.

Sonically treated DNA (10 $\mu\text{g}/\text{ml}$ of $0.1 \times \text{SSC}$ [$1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M trisodium citrate] [pH 7.0]) was denatured by heating at 110°C for 10 min and then rapidly chilled on ice. The procedure used for DNA-DNA hybridization was that described by Stackebrandt and Kandler

(29). Incubation was performed for 24 h at 47°C in a water bath (Salvis) with gentle reciprocal agitation (25 movements per min) to reduce the possibility of a concentration gradient of radioactive DNA fragments being formed as described by Johnson and Ordal (20).

Each reaction was run in sextuple. Negative controls (filters bearing calf thymus DNA) were included for each labeled DNA tested.

At the end of the incubation period, the reaction mixtures were removed, and the filters were washed twice with cold $3 \times \text{SSC}$ and dried in a vacuum oven at 70°C. For each hybridization test, the reaction mixture, washed solutions, and filter were counted by using a model SL 4000 liquid scintillation system (Intertechnic, Kontron S.A.) and a pseudocumene-based scintillation fluid (HP/b; Beckman Instruments, Inc., Fullerton, Calif.). For each test, the proportion of radioactive DNA bound to filter DNA was estimated, and then the DNA homology values were calculated (mean percentage of heterologous binding compared with homologous binding).

Enzymes, standard DNA, and radioisotopes. Proteinase K was purchased from E. Merck AG, Darmstadt, Federal Republic of Germany. Lysozyme, ribonuclease A, calf thymus DNA (type I), *E. coli* B DNA (type VIII) and *M. lysodeikticus* DNA (type XI) were obtained from Sigma Chemical Co., St. Louis, Mo. [^3H]thymidine (25 Ci/mmol) was purchased from C.E.A., Saclay, France.

RESULTS

The enrichment and isolation procedure yielded a pure culture of a cellulolytic organism (strain M^T). Colonies of the isolate on cellulose agar measured about 1.5 mm after 7 days of incubation at 30°C. After 2 weeks, these colonies were circular, approximately 2 to 3 mm in diameter, and white, and they had an undulate margin. Zones of 1 to 2 mm of cellulose digestion extending from the colony periphery were clearly visible.

The microscopy studies showed that the isolate is a rod-shaped, coryneform, nonmotile bacterium occurring mostly singly and in pairs (V forms) and exhibiting a polymorphism: club-shaped rods and rods of various lengths (Fig. 1A).

Cells in the exponential growth phase were gram negative (36), but transmission electron microscopy of thin sections of this strain showed the cell wall profile and ultrastructural details typical of gram-positive-type organisms (Fig. 1B). It was therefore possible to estimate the dimensions of the cells: 0.2 to 0.5 by 0.6 to 1.7 μm .

Growth of the new isolate was similar under both anaerobic and aerobic conditions with shaking on 0.2% cellobiose. Under both conditions, the biomass increase was similar (change in absorbance at 450 nm: 1.5 and 2.0, respectively), and fermentation products accumulated. The

FIG. 1. Electron micrographs of cells of strain M^T grown on basal medium supplemented with 0.2% cellobiose for 2 days. (A) Negative staining; bar = 1 μm . (B) Thin section; bar = 0.1 μm .

three main fermentation products detected under anaerobic conditions (acetate, formate, and ethanol) were also estimated in the culture supernatant from aerobic growth (Table 1). The results seem to indicate that this strain does not have a classical oxydative metabolism and derives its energy essentially from fermentation metabolism under aerobic growth conditions.

Yeast extract at the minimum concentration of 0.05% was necessary for optimum growth. It could be replaced by Casamino Acids (0.2% [wt/vol]) and, less efficiently, with a vitamin cocktail. A mixture of biotin and thiamine was not sufficient to ensure growth.

The isolate grew under both aerobic and anaerobic conditions on a wide variety of substrates, including cellulose, cellobiose, glucose, and raffinose, but not on ribose, and it utilized acetate under aerobic conditions but not lactate and gluconate. (The extensive list of nutrients used is given in the taxonomic description at the end of the discussion.)

The metabolic products detected in cellulose culture after 15 days of fermentation were acetic acid, formic acid, and ethanol. In addition, carbon dioxide and succinic acid were produced in smaller amounts, and occasionally L-(+)-lactic acid. No acetylmethylcarbinol was produced, and no H_2 was evolved. Strain M^T produced NH_3 from peptone, nitrate reductase, cytochrome oxidase, gelatinase, and β -galactosidase but failed to produce indole, catalase, or urease.

Alanine and ornithine are present in the strain M^T cell wall, and its main sugar component is glucose, but rhamnose and ribose are also present.

The DNA base composition is 75.8 mol% G+C (mean from two experiments).

The isolate showed a DNA homology of 37 to 40% with

TABLE 1. Comparison of growth on cellobiose (2 g/liter) under anaerobic and aerobic conditions

Culture condition	ΔA_{450}^a	Fermentation product (mol/mol of hexose consumed)		
		Acetate	Formate	Ethanol
Anaerobic	1.5	0.73	0.90	0.37
Aerobic	2.0	0.44	0.16	0.04

^a A_{450} , Absorbance at 450 nm.

Cellulomonas sp. ATCC 21399 (DSM 20108) which was assimilated to *C. uda* (Kellermann et al. [3]) by Stackebrandt and Kandler (28) (Table 2).

DISCUSSION

Based on its morphological and other characteristics, the gram-positive, nonsporeforming, coryneform, cellulose-decomposing bacterium described herein belongs to the family *Corynebacteriaceae* (8). Several studies have been performed with a view to characterizing the various genera of this family by using various approaches, including numerical taxonomy studies (5, 11, 12, 34). They have led to contradictory results, and some cellulolytic strains have been classified as belonging to genera other than *Cellulomonas*.

Studies on the *Cellulomonas* genus subsequently showed that certain characters such as the peptidoglycan type of the cell wall (13), the G+C content of the DNA (37), the fermentation pattern (28), serological properties (6), isoprenoid quinones (10, 38), and DNA homology (27, 29) demonstrated the homogeneity of this genus. Differences between the various known strains have clearly demonstrated the existence of eight species in this genus (24, 26), despite the fact that only one species was recognized in the 1974 edition of Bergey's manual (8).

From these studies, it appears that the most salient features defining members of the genus *Cellulomonas* are its cellulolytic aptitude and the high G+C content of its DNA (71 to 77 mol%). The new isolate fulfilled both these criteria. In addition, its DNA homology (37 to 40%) with a strain of *Cellulomonas* sp. confirmed that it belongs to this genus.

Indeed, it can be seen (Table 2) that the range of DNA homologies between several established species of *Cellulomonas* is 12 to 50%. However, in contrast with previously described *Cellulomonas* species, aerobiosis did not offer any energetic advantage for its growth, and the strain was thought to probably derive its energy essentially from fermentation processes although placed under aerobic conditions. Furthermore, the combined morphological and biochemical characteristics of this organism (especially the cell wall sugars and the utilization of certain carbon sources, as pointed out by Stackebrandt and Kandler [29]), do not match the definitions of any of the known species (Table 3). Biotin and thiamine were not the sole growth-promoting factors required by the strain as commonly reported for *Cellulomonas* strains (8). Nevertheless, similar growth requirements were found for several *Cellulomonas* strains (9, 33) and *C. uda* ATCC 21399 (14), in which small amounts of yeast extract added to the basal medium greatly enhanced growth.

We therefore propose the establishment of a new species, *Cellulomonas fermentans*, sp. nov. (Fer. men'tans. L. part. adj. *fermentans* fermenting) with the following specific description.

Morphology. Coryneform rod exhibiting a polymorphism, 0.2 to 0.5 by 0.6 to 1.7 μ m. Nonmotile. Gram positive.

Colony characteristics. White with an undulate margin, 2 to 3 mm in diameter. A zone of cellulose digestion extending from the periphery of the colony is observed in cellulose agar.

Physiology. Anaerobic, but with growth under aerobic conditions without any apparent benefit. Growth on arabinose, cellulose, fructose, galactose, glucose, glycogen, lactose, maltose, mannose, mannitol, raffinose, starch, sucrose, trehalose, xylose, and xylan but not on asparagine, arbutine, glycerol, inulin, melibiose, melezitose, pectin, rhamnose, ribose, pyruvate, salicin, sorbitol, and urea. It does not grow under aerobic conditions on butanol, butyrate, citrate, ethanol, gluconate, lactate, propanol, propionate, or succinate but grows slowly on acetate. Yeast extract (0.05%) and Casamino Acids (0.2%) support only slight growth. Growth factors other than biotin and thiamine together are required and can be provided by 0.05% yeast extract.

TABLE 2. DNA homology between the new isolate and *C. uda* ATCC 21399 compared with DNA homologies among various *Cellulomonas* spp.

Source and strain	[³ H]DNA homology (%) ^a with filter DNA from	
	<i>C. uda</i> ATCC 21399	New isolate
Present study		
<i>C. uda</i> ATCC 21399	100	37
New isolate	40	100
Reference 29		
<i>C. uda</i> ATCC 491 ^T	90	
<i>C. fimi</i>	34-39	
<i>C. biazotea</i>	27	
<i>C. gelida</i>	43-49	
<i>C. cartae</i>	12	
<i>C. cellasea</i>	27	

^a Homology index is expressed as the percentage (mean of six experiments) of [³H]DNA bound to a particular filter DNA, relative to the homologous reaction.

TABLE 3. Physiological data and G+C contents of the DNAs of *Cellulomonas* species^a compared with those of the new isolate

Species	Motility	Cell wall diamino acid	Cell wall sugars					Ni-trate reduction	NH ₃ from peptone	Utilization of:				G+C (mol%)	Colony pigment
			Glucose	Glucose	Rhamnose	Fucose	Ribose	6-Desoxyxylitolose	Galactose	Ribose	Raffinose	Acetate	L(+)-Lactate		
<i>C. biazotea</i>	+	Orn	+	+	+	+	+	+	+	+	+	+	+	71.5-75.6	Yellow-white
<i>C. cellasea</i>	-	Orn	-	-	+	+	+	+	+	+	+	+	+	75.0	Yellow
<i>C. fimi</i>	+	Orn	+	+	+	+	+	+	+	+	+	+	+	71.3-72.0	Yellow-white
<i>C. flavigena</i>	-	Orn	+	+	+	+	+	+	+	+	+	+	+	72.7-74.8	Yellow
<i>C. gelida</i>	+	Orn	+	+	+	+	+	+	+	+	+	+	+	72.4-74.4	Yellow-white
<i>C. uda</i>	-	Orn	+	+	+	+	+	+	+	+	+	+	+	72.0-76.3	White
<i>C. cariae</i> ^b	+	Lys	0 ^c	0	+	+	+	+	+	+	+	+	+	72.0-76.6	Yellow-white
<i>C. turbata</i>	+	Lys	+	+	+	+	+	+	+	+	+	+	+	70.5-75.0	Yellow
New isolate	+	Orn	+	+	+	+	+	+	+	+	+	+	+	75.8	White

^a According to references 22, 27, 29, 30, and 31.

^b *C. cariae* can be easily distinguished from other species of *Cellulomonas* by its ability to hydrolyze xanthine.

^c 0, Not furnished.

Fermentation products. Fermentation products are acetic acid, formic acid, ethanol, occasionally L-(+)-lactic acid, and, in smaller amounts, carbon dioxide and succinic acid.

Biochemical reactions. Gelatin liquefied, nitrate reduced, NH_3 produced from peptone. Neither acetyl methyl carbinol nor indole is produced. Catalase and urease negative.

Temperature relationships. Optimum temperature between 30 and 37°C. No growth at 50°C.

pH relationships. Optimum pH around 7.4. Growth rate decreases substantially under 7 and over 8.

Cell wall structure. Ornithine is the diamino acid of the peptidoglycan. Cell wall sugars are glucose, rhamnose, and ribose.

DNA base composition. 75.8 mol% G+C.

Source. Methanogenic enrichment culture from a municipal dumping ground was the source.

Type strain. Strain M^T. This strain has been deposited in the Deutsche Sammlung von Mikroorganismen (DSM) under accession number 3133.

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