Clostridium ljungdahlii sp. nov., an Acetogenic Species in Clostridial rRNA Homology Group I

RALPH S. TANNER,^{1*} LETRISA M. MILLER,¹ AND DECHENG YANG²

*Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019,¹ and
Department of Microbiology, University of Illinois, Urbana, Illinois 61801²*

Clostridium ljungdahlii sp. nov. strain ATCC 49587^T (T = type strain) was isolated from chicken yard waste for its ability to produce ethanol from synthesis gas. This gram-positive, motile, sporeforming rod's metabolism was primarily acetogenic. *C. ljungdahlii* grew with carbon monoxide, hydrogen and carbon dioxide, ethanol, pyruvate, arabinose, xylose, fructose, or glucose. Methanol, ferulic acid, lactate, galactose, and mannose did not support growth. The G+C content was 22 to 23 mol%. *C. ljungdahlii* is the first acetogen in clostridial 23S rRNA homology group I.

The potential for microbial production of liquid fuels (e.g., ethanol) and commodity chemicals (e.g., acetic acid) from synthesis gas (CO-H₂-CO₂) derived from coal has been recognized (27) and has prompted investigations of microorganisms which can metabolize the components of synthesis gas under anoxic conditions. A strain which could produce ethanol from synthesis gas was isolated from chicken yard waste (2, 25). In this report we describe this strain, a new, anaerobic, gram-positive, spore-forming rod which is the first acetogenic species in clostridial rRNA group I, as described by Johnson and Francis (15).

(A portion of this work has appeared previously [24].)

MATERIALS AND METHODS

The acetogenic clostridium described here was isolated by Sudhakar Barik at the University of Arkansas from an enrichment inoculated with chicken yard waste at an initial pH of 5.0 and incubated at 37°C under an atmosphere of synthesis gas (CO-H₂-CO₂-CH₄, 73:15:10:2) (2). This isolate was named *Clostridium ljungdahlii*. The single strain was designated strain PETC^T (T = type strain) and was deposited in the American Type Culture Collection as strain ATCC 49587^T.

Media. The media used for routine growth and characterization experiments were prepared by using strict anoxic techniques (1) and contained (per liter) 1.0 g of NH₄Cl, 0.8 g of NaCl, 0.1 g of KCl, 0.1 g of KH₂PO₄, 0.2 g of MgSO₄ · 7H₂O, 0.02 g of CaCl₂ · 2H₂O, 1.0 g of NaHCO₃, 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.2 g of cysteine hydrochloride, 0.2 g of Na₂S · 9H₂O, 10 ml of a trace metal solution (22), and 10 ml of a vitamin solution (22). An atmosphere containing N₂ and CO₂ (80:20, pressurized to 70 kPa) was used for growth with organic substrates, which were added to a final concentration of 5 g/liter. H₂-CO₂ (80:20, pressurized to 200 kPa) or CO-N₂-CO₂ (75:20:5, pressurized to 140 kPa) was used for growth on gaseous substrates. The initial medium pH was 5.8 to 5.9. Cultures were incubated at 37°C. The optimum pH (initial pH) was determined in medium containing fructose as the substrate and sodium acetate, 2-(*N*-morpholino)ethanesulfonic acid (MES), or *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (Sigma Chemical Co., St. Louis,

Mo.) as a buffer (1.0 g/liter). The optimum temperature for growth was determined by using fructose as the substrate.

Fermentation balance. A fermentation balance was determined for growth on fructose or H₂-CO₂. Fructose was measured by using a phenol-sulfuric acid carbohydrate assay (9). Utilization of H₂-CO₂ (80:20) was determined by using a pressure transducer system (8) to measure the reduction of gas pressure in sealed culture tubes (1). Individual gases were measured with a gas chromatograph equipped with a thermal conductivity detector (Varian, Sugar Land, Tex.) and a Porapak Super Q column (Alltech, Deerfield, Ill.). The following equation was used to calculate the amount of gas consumed: 4H₂ + 2CO₂ → CH₃COOH + 2H₂O (19). Acetic acid was measured by ion-exclusion high-performance liquid chromatography on an Aminex HPX-87H column (Bio-Rad, Richmond, Calif.). Ethanol was measured enzymatically with alcohol dehydrogenase (kit 332-B; Sigma) or by gas chromatography on a GP 10% SP-1200/1% H₃PO₄ Chromosorb WAW column (Supelco, Inc., Bellefonte, Pa.).

Electron microscopy. Cells of fructose- or H₂-CO₂-grown *C. ljungdahlii* were allowed to settle onto carbon-coated Formvar film on copper electron microscope grids. For whole-cell transmission electron microscopy, cells were fixed with glutaraldehyde or left untreated and then negatively stained with phosphotungstic acid or uranyl acetate or left unstained. Electron micrographs were obtained with a Zeiss model EM-10 transmission electron microscope. Energy-dispersive spectroscopy (12) of unstained fructose- or H₂-CO₂-grown cells was performed with a JOEL model 2000 transmission electron microscope equipped with a Delta 4 Quantum thin-window X-ray detector (Kevex, San Carlos, Calif.).

16S rRNA sequence analysis. Cells of *Clostridium barkeri* ATCC 25849, *Clostridium lituseburense* ATCC 25759, *Clostridium pasteurianum* ATCC 6013, and *Clostridium tyrobutyricum* ATCC 25755 were provided by John L. Johnson. The 16S rRNA sequences of *C. barkeri*, *C. lituseburense*, *C. ljungdahlii*, *C. pasteurianum*, and *C. tyrobutyricum* were determined in the laboratory of Carl R. Woese (17, 20). The 16S rRNA sequences of *Bacillus subtilis* and *Escherichia coli* have been published previously (3, 13). Sequences were analyzed by a distance matrix analysis by using a program for fitting trees to distance matrix data, as adapted by workers in the laboratory of Carl R. Woese (7, 16, 20).

Analytical procedures. Growth was determined by measuring turbidity in aluminum seal tubes (1). DNA from *C. ljungdahlii* was purified, and the G+C content was deter-

* Corresponding author.

FIG. 1. Transmission electron micrograph of *C. ljungdahlii*. Cells were spread on a carbon-coated Formvar grid and negatively stained with 1% phosphotungstic acid (pH 7). The micrograph was taken with a Zeiss model EM-10 transmission electron microscope.

mined from the melting temperature by using a Beckman model DU-8B spectrophotometer equipped with a melting temperature module (14). Indole production and esculin hydrolysis were determined by using standard assays (21).

Nucleotide sequence accession numbers. The nucleotide sequences of *C. barkeri*, *C. lituseburense*, *C. ljungdahlii*, *C. pasteurianum*, and *C. tyrobutyricum* were deposited in the GenBank library under accession numbers M23927, M59107, M59097, M23930, and M59113, respectively.

RESULTS AND DISCUSSION

Cellular morphology. Electron and phase-contrast microscopy revealed that cells of *C. ljungdahlii* were motile, straight rods (0.6 by 2 to 3 μm) occurring mostly as single cells (Fig. 1). Spores were rarely observed; terminal to subterminal nonswelling structures tentatively identified as forespores were observed infrequently. Cells were peritrichous and had a thick (0.1- to 0.2- μm) coat of material of unknown composition surrounding each cell. Electron microscopy of *C. ljungdahlii* was problematic. The majority of cells in unstained or stained specimens that were not fixed or were fixed with glutaraldehyde were electron opaque. Energy-dispersive spectroscopy (Fig. 2) indicated that these electron-opaque cells contained calcium, as shown by the X-ray peaks at 3.7 keV ($K\alpha_1$) and 4.0 keV (K edge) (12). The calcium was probably associated with the cell envelope, since lysed cells also were frequently opaque to transmission electron microscopy (data not shown).

Physiology. *C. ljungdahlii* grew autotrophically with H_2 - CO_2 or CO and used a number of organic compounds, including ethanol, pyruvate, and some simple carbohydrates, as carbon and energy sources (Table 1). Growth on formate was poor. Cultures partially metabolized malate, as indicated by a pH change in the medium after incubation with this substrate. Improved control of culture pH near the

optimum value (see below) might result in better growth of *C. ljungdahlii* with formate or malate. Methanol, even when tested at a concentration of 1 g/liter, as well as the methoxylated compounds ferulic acid and trimethoxybenzoate, did not support growth.

Addition of a vitamin solution (22) was required for growth of *C. ljungdahlii*. Addition of yeast extract (1 g/liter) or Casamino Acids (2 g/liter) was also required for reliable, reproducible growth. The exact nutritional requirements of *C. ljungdahlii* were not determined.

The optimum initial culture pH for growth was 6.0; the initial culture pH range at which growth occurred was 4.0 to 7.0. A final pH of 3.9 to 4.1 was obtained when *C. ljungdahlii* was cultured in unbuffered media. The optimum temperature for growth was 37°C; the growth temperature range was 30 to 40°C. Under the optimal pH and temperature conditions, a doubling time of 0.26 h^{-1} was obtained for growth with either fructose or H_2 - CO_2 .

Cultures of *C. ljungdahlii* quantitatively converted 4 mmol of hydrogen and 2 mmol of carbon dioxide into 1 mmol of acetic acid or 1 mmol of fructose into 2.44 mmol of acetic acid; no other end products of metabolism were detected in these experiments. The fermentation balances demonstrate that *C. ljungdahlii* is an acetogen, a bacterium which can synthesize acetate from one-carbon compounds (19). Most acetogens quantitatively convert carbohydrates to acetate: 1 hexose $\rightarrow$ 3 acetic acid. The conversion by *C. ljungdahlii* of 1 fructose molecule to 2.44 acetate molecules is similar to the yields of acetate observed for other acetogens: 2.1 to 2.3 acetate molecules per hexose molecule for *Acetobacterium carbinolicum* (10); 2.2 to 2.8 acetate molecules per fructose molecule for *Acetobacterium woodii* in media supplemented with yeast extract and/or Trypticase (22a); 2.3 to 3.0 (average, 2.5) acetate molecules per glucose molecule for *Acetogenium kivui* (18); an average of 2.55 acetate molecules per

FIG. 2. X-ray spectrum from a cell of *C. ljungdahlii*.TABLE 1. Substrate utilization by *C. ljungdahlii*

Substrate ^a	Result ^b	Substrate	Result
H ₂ -CO ₂	+	Ribose	+
CO	+	Xylose	+
Sodium formate	+/-	Glucose	+ ^d
Methanol	-	Fructose	+
Ethanol	+	Galactose	-
Sodium pyruvate	+	Mannose	-
Sodium lactate	-	Sorbitol	-
Glycerol	-	Sucrose	-
Sodium citrate	-	Lactose	-
Sodium succinate	-	Maltose	-
Sodium fumarate	+	Starch	-
Malic acid	- ^c	Ferulic acid	-
Erythrose	+	Trimethoxybenzoic acid	-
Threose	+	Casamino Acids	+/-
Arabinose	+	Alanine	-

^a Each substrate was tested for the ability to support growth of *C. ljungdahlii*. Gaseous substrates were added to the gas phase of a crimp-sealed tube. Other substrates were added at a concentration of 5 g/liter to a medium containing 1 g of yeast extract per liter. A 2% inoculum of fructose-grown cells was used; no growth or poor growth results were confirmed by using H₂-CO₂-grown cells as an inoculum.

^b Growth was measured in each aluminum seal tube (1). Levels of growth (compared with the control) were scored as follows: +, A_{600} of >0.1; +/-, A_{600} between 0.1 and 0.01; and -, A_{600} of <0.01.

^c Malate was metabolized by *C. ljungdahlii*, as indicated by a change in the culture pH.

^d Cultures grown previously on fructose or H₂-CO₂ required adaptation for growth on glucose.

glucose molecule for *Clostridium thermoaceticum* (11); and 2.5 acetate molecules per glucose molecule for *Clostridium thermoautotrophicum* (26). When, examined, the balance of the hexose utilized was converted into cell mass (10). In the strictest sense, an acetogen is a bacterium that uses the Wood-Ljungdahl pathway to reduce CO₂ to acetate (19). The quantitative conversion of CO₂ and H₂ to acetic acid by *C. ljungdahlii* is consistent with the probability that this organism possesses this pathway.

Cultures of *C. ljungdahlii*, which was isolated for its ability to convert synthesis gas to ethanol (2, 25), often have 0 to 2 mM ethanol present as an end product of metabolism. The ability to both produce and consume ethanol has been observed in other acetogens (specifically, in species of the genus *Acetobacterium*) (4). Cultures may also produce 0 to 0.3 CO₂ molecule per fructose molecule fermented. Vega et al. (25) noted that *C. ljungdahlii* produced trace amounts of CO₂ when it fermented fructose and produced CO₂ and traces of H₂ when it metabolized CO.

Cultures did not hydrolyze esculin and produced indole from tryptophan.

Phylogeny. The phylogenetic relationship of *C. ljungdahlii* to other clostridia is shown in Fig. 3. *C. ljungdahlii* was found to be closely related to *C. tyrobutyricum*, which ferments carbohydrates to butyrate and acetate (5). *C. ljungdahlii* is a member of clostridial 23S rRNA homology group I; the 16S rRNA sequence analysis clustered *C. ljungdahlii* with two other group I clostridia, *C. pasteurianum* and *C. tyrobutyricum*. *C. lituseburens* is a species in clostridial rRNA homology group II, and *C. barkeri* is a representative of the clostridia whose DNAs have compar-

FIG. 3. Phylogeny of *C. ljungdahlii* as determined from a 16S rRNA sequence analysis. The total horizontal distance between two species indicates the difference between their sequences. The bar indicates a distance corresponding to a 5% difference.

atively high G+C contents and which do not belong to either rRNA homology group, as described by Johnson and Francis (15). *C. ljungdahlii* is the first known acetogen in clostridial rRNA homology group I. The phylogeny of six other acetogens (*Acetobacterium woodii*, *Acetogenium kivui*, *Clostridium aceticum*, *Clostridium formicoaceticum*, *C. thermoaceticum*, and *Eubacterium limosum*) was examined by 16S rRNA oligonucleotide cataloging, which showed that none of these species belonged to either homology group I or group II of the clostridia (23). A more recent examination of the phylogeny of the clostridia, including the rRNA homology groups, has been published (6).

C. ljungdahlii's DNA had a G+C content of 22 to 23 mol%, as determined by the melting temperature method. *Clostridium putrefaciens* is another clostridium with a very low G+C content (22 to 25 mol%) reported for its DNA (5). This G+C content is significantly lower than the G+C contents reported for other acetogens (19).

Phenotypically, *C. ljungdahlii* is distinguishable from most clostridia since its metabolism is basically acetogenic. *C. ljungdahlii* is phenotypically distinct from other acetogens as follows (5, 19): from *Acetogenium kivui*, *C. thermoaceticum*, and *C. thermoautotrophicum*, which are thermophiles; from *Acetobacterium carbinolicum*, *Acetobacterium woodii*, *C. formicoaceticum*, *C. thermoaceticum*, *C. thermoautotrophicum*, and *Eubacterium limosum*, which can grow with methanol; from *C. formicoaceticum* and *Clostridium magnum*, which do not grow with H_2 - CO_2 ; from *C. thermoaceticum* and *Eubacterium limosum*, which are not motile; from *Acetobacterium carbinolicum*, *Acetobacterium wieringae*, and *Acetobacterium woodii*, which can grow with lactate; and from *C. aceticum*, whose pH optimum is 8.3. The optimum growth pH is lower than that reported for most acetogens (19); only *C. thermoautotrophicum* has a lower reported optimum pH (pH 5.7).

C. ljungdahlii is the first known acetogenic member of rRNA homology group I, which includes solvent-producing species, such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and *Clostridium butyricum* (5, 15). A comparative biochemical study of these species could be helpful for improving the conversion of synthesis gas to ethanol catalyzed by *C. ljungdahlii* (2, 25).

Description of *Clostridium ljungdahlii* sp. nov. *Clostridium ljungdahlii* (ljung.dahl'i.i. M.L. gen. n. *ljungdahlii*, of Ljungdahl, in recognition of Lars G. Ljungdahl's research contributions in both the study of acetogens and the study of clostridia). Cells are gram-positive, motile rods (0.6 by 2 to 3 μ m) and occur mostly singly. Cells rarely sporulate. Growth occurs only under anoxic conditions. The optimum temperature and initial pH for growth are 37°C and 6.0, respectively.

Grows autotrophically with H_2 - CO_2 or CO and chemolithotrophically with formate, ethanol, pyruvate, fumarate, erythrose, threose, arabinose, xylose, glucose, and fructose. Methanol, ferulate, trimethoxybenzoate, lactate, glycerol, citrate, succinate, galactose, mannose, sorbitol, sucrose, lactose, maltose, and starch are not utilized. Acetic acid is the major end product of metabolism; traces of ethanol may also be produced.

The G+C content of the DNA is 22 to 23 mol% (as determined by the melting temperature method). A member of clostridial 23S rRNA homology group I, as determined by 16S rRNA sequence analysis.

The type strain, strain PETC, was isolated from chicken yard waste and has been deposited in the American Type Culture Collection as strain ATCC 49587.

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