

## *Acetobacterium*, a New Genus of Hydrogen-Oxidizing, Carbon Dioxide-Reducing, Anaerobic Bacteria

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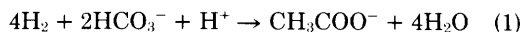
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A new genus of fastidiously anaerobic bacteria which produce a homoacetic fermentation is described. Cells are gram-positive, oval-shaped, short rods which are actively motile by means of one or two subterminal flagella. Hydrogen is oxidized, and carbon dioxide is reduced to acetic acid. Organic substrates which are fermented in a mineral medium include fructose, glucose, lactate, glycerate, and formate. Pantothenate is required as a growth factor. The deoxyribonucleic acid base composition of the type species is 39 mol% guanine plus cytosine. The name *Acetobacterium* is proposed for this new genus, which is tentatively placed in the family *Propionibacteriaceae*. The type species, *Acetobacterium woodii* sp. nov., is named in honor of Harland G. Wood. The type strain of *A. woodii* is WB1 (= ATCC 29683 and DSM 1030).

Homoacetic fermentations of carbohydrate by anaerobic bacteria are rare. For a number of years, Wood and his associates studied the total synthesis of acetate from carbon dioxide in the homoacetic fermentation of glucose by *Clostridium thermoaceticum* (5, 8, 14, 15). Results of recent work indicate that in the 1 mol of acetate which is synthesized from 2 mol of carbon dioxide by this organism, the carboxyl group of pyruvate rather than free carbon dioxide is the precursor of the carboxyl group of acetate by a transcarboxylation reaction (14). *Clostridium formicoaceticum* likewise produces a homoacetic fermentation of fructose (1, 11). These organisms require reduced organic compounds as substrates, and neither organism produces nor uses molecular hydrogen.

The oxidation of hydrogen and the reduction of carbon dioxide to acetic acid by a stabilized enrichment culture were reported in 1932 (6); a few years later, the isolation of *Clostridium acetium* was accomplished by Wieringa (16, 17). This organism produced acetic acid from hydrogen and carbon dioxide as well as from carbohydrates. Unfortunately, *C. acetium* was lost soon after the third paper on this anaerobe was published in 1948 (7). Repeated attempts to reisolate it in various laboratories over the past 25 years have failed.

We report here the isolation of an organism which produces a homoacetic fermentation and which grows by the anaerobic oxidation of hydrogen and reduction of carbon dioxide according to the following equation:



The  $\Delta G'_0$  of this reaction is  $-25.6$  (4). To our knowledge, this is the first organism to be isolated, since the loss of *C. acetium*, which is capable of reducing carbon dioxide to acetic acid by the use of molecular hydrogen. A brief report on this organism has appeared previously (S. M. Schoberth and W. E. Balch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, I90, p. 131).

### MATERIALS AND METHODS

**Bacterial strains.** Strain WB1 of the acetogenic organism was isolated from black sediment of Oyster Pond Inlet, a marine estuary, Woods Hole, Mass. Strains RT1 and RT2 were obtained from black sediments of Crystal Lake, Urbana, Ill.

**Media.** Sterile media for the isolation, subculture, and maintenance of organisms were prepared by techniques recently described (2). Basal medium (M1) contained (in grams per liter of distilled water):  $\text{NH}_4\text{Cl}$ , 1.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1;  $\text{K}_2\text{HPO}_4$ , 0.4;  $\text{KH}_2\text{PO}_4$ , 0.4; resazurin, 0.0001; cysteine · HCl, 0.5;  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ , 0.5;  $\text{NaHCO}_3$ , 2.0. Vitamin solution and mineral solution (18) were each added in 10-ml amounts to 1 liter of medium. The final pH of the medium, after sparging with nitrogen or hydrogen which contained 20% carbon dioxide, was 6.7. When medium M1 was incubated in a pressurized atmosphere of  $\text{H}_2\text{-CO}_2$  (67:33) of 2 atm,  $\text{NaHCO}_3$  was added at a concentration of 7 g per liter. Solid medium (M2) for plates was prepared by the addition of the following compounds (grams per liter) to medium M1: Noble agar (Difco), 20.0;  $\text{CaCO}_3$ , 10.0; and yeast extract (Difco), 2.0. Plates were poured and streaked in an anaerobic chamber and were then incubated under a gas mixture of  $\text{H}_2\text{-CO}_2$  (67:33) at a pressure of 2 atm by the use of techniques which we have recently described in detail (2). Organic substrates (see Table 1) were added at a final concen-

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tration of 0.5% (wt/vol) to either medium M1 or M2 as desired.

**Methods.** Growth of the organism in each culture tube (optical path 18 mm) was followed by measuring the absorbancy in a Bausch and Lomb spectrophotometer model 20 at 660 nm. Gas consumption by the organism was determined by measurement at appropriate intervals during growth of the pressure inside each culture tube (2). Cells were mass-cultured in fermentors by techniques reported previously (3); the fermentor contained a pH electrode, and sterile NaOH was automatically added to maintain the pH at 6.7. The production of acetate was determined by the acetate kinase method (12), and the utilization of fructose was determined by the method of Nelson (10). A Packard gas chromatograph equipped with a Poropak Q100-120 mesh column and an electron-capture detector was used to quantitate hydrogen and carbon dioxide.

To study the stoichiometry of H<sub>2</sub> oxidation and CO<sub>2</sub> reduction to acetic acid, growth of cultures was followed in sealed tubes in medium M1. Each tube was pressurized to 2 atm with a mixture of H<sub>2</sub>-CO<sub>2</sub> (67:33). An inoculum which represented 2% of the medium volume was added to tubes in which growth was to be followed; uninoculated tubes served as controls to determine possible gas loss through leakage during sampling. At each time period, a 100- $\mu$ l sample of medium was removed with a syringe and was assayed for acetate. The gas pressure in each tube was measured, and gas consumption was calculated (2). Acetic acid which was formed during growth was neutralized by injection of sterile 0.5 M NaOH.

For the determination of the vitamin requirements of the acetogenic organism, medium M1 was prepared without the addition of the vitamin solution, and NaHCO<sub>3</sub> (7 g/liter) was added. A 5-ml amount of medium was placed in each tube, and sealed tubes were pressurized to 2 atm with a gas mixture of H<sub>2</sub>-CO<sub>2</sub> (67:33).

For isolation of deoxyribonucleic acid (DNA), cells were suspended at a concentration of 0.4 g/ml in 0.2 M ethylenediaminetetraacetate (pH 8) which contained 0.075 M NaCl and were passed through a French pressure cell at 3,000 lb/in<sup>2</sup>. Sodium dodecyl sulfate was added to the cell lysate at a final concentration of 2%, and the DNA was purified from the lysate by the method of Marmur (9) with the following modifications: (i) the lysate was incubated at 37°C overnight with 3 mg of Pronase (Calbiochem, La Jolla, California) per ml prior to the addition of sodium perchlorate; (ii) after the pancreatic ribonuclease digestion of the DNA preparation, the digest was deproteinized using water-saturated phenol. The buoyant density of the DNA was determined by a cesium chloride density gradient centrifugation in a Beckman model E ultracentrifuge. The moles percent guanine plus cytosine (G+C) was calculated by the method of Schildkraut et al. (13). DNA from *Micrococcus luteus* was used as a standard.

For electron microscopy, cells were negatively stained with a 3% aqueous solution of uranyl acetate, pH 4.8. Micrographs were taken with a Philips EM 301 microscope.

## RESULTS

**Development of enrichment-culture techniques.** In the studies reported here, evidence for hydrogen-oxidizing acetogenic bacteria was first observed in an enrichment culture for hydrogen-oxidizing methanogenic bacteria. The culture was initiated by inoculation of 0.5 ml of black lake sediment into 8 ml of medium M1 in a tube (18 by 150 mm) fitted with a solid black rubber stopper pierced by two 20-gauge hypodermic needles, one of which reached to the bottom of the tube and the other of which, being only 2 cm long, did not. A mixture of H<sub>2</sub>-CO<sub>2</sub> (80:20) was passed through the longer needle at a rate of about 0.5 cm<sup>3</sup>/min, and the effluent was vented through tubing from the short needle so that it bubbled through water in an open tube. To insure a more efficient use of gases, several tubes were connected in series by polyethylene tubing so that the effluent of one tube became the influent for the next. After 3 weeks of continuous bubbling at room temperature, no methane was detected in the effluent, but acetic acid was readily detectable. The black rubber stoppers were swollen and deformed, exhibiting the typical response of such stoppers when they are exposed to vapors of acetic acid. The turbidity of the medium was negligible, and we were amazed that such a small biomass could produce acetic acid in an amount sufficient to produce the above-mentioned results.

With black sediment from a marine estuary, the experiment described above was repeated with similar results. After 4 weeks of bubbling with the mixture of H<sub>2</sub> and CO<sub>2</sub>, the pH of the medium was 5.0. Results of a gas-chromatographic analysis of the medium indicated that a single product, acetic acid, was produced in a concentration which reached 0.06 M. Microscopic examination of the growth liquor revealed a dominant population of oval-shaped, highly motile cells frequently occurring in pairs.

When enrichment cultures were attempted in a closed system, growth of methanogenic organisms interfered with the isolation of the acetogenic bacteria. One of us (R.T.) devised a selective medium in which the addition of sodium dithionite inhibited the growth of methanogenic bacteria. *Methanobacterium ruminantium* strain M-1, for example, was inhibited at a concentration of 5 mg/100 ml of medium, whereas the acetogenic bacteria were resistant to concentrations as high as 40 mg/100 ml. A successful enrichment was obtained by placing a 5-ml sample of black sediment in a 100-ml serum bottle, which was then sealed and flushed with hydrogen. A 5-ml amount of medium M1 which con-

tained 1 mg of sodium dithionite was injected into the sealed bottle. By use of techniques recently described (2), an atmosphere of  $H_2$ - $CO_2$  (67:33) was placed in the bottle to a final pressure of 1.7 atm. Each bottle was incubated at 30°C without shaking, since for unknown reasons agitation of the culture inhibited growth. Each culture bottle was repressurized every 3 days until the production of acetic acid reached the 100 mM level.

**Isolation of bacteria.** A sample from a successful enrichment culture, which had been inoculated with sediment from a marine estuary, was streaked on medium M2, and plates were incubated at 30°C as previously described (2). Acetogenic colonies were distinguished by a clearing of the  $CaCO_3$  in the vicinity of each colony (Fig. 1). Circular, convex colonies were usually visible after a few days and reached a diameter of 1 mm in 7 to 10 days. Older colonies showed a slight yellow pigmentation; however, no fluorescence was observed under ultraviolet light either under anaerobic or aerobic condi-

tions. When tested, the colonies did not give a positive catalase reaction. The organism (designated strain WB1) proved to be a strict anaerobe, and it was cloned, maintained in stock culture, and subcultured by a modification of the Hungate technique (2). Colonies were composed of nonsporeforming, motile, oval-shaped rods which frequently occurred in pairs. The rods measured about 1  $\mu m$  in width and about 2  $\mu m$  in length and contained a single, long, subterminal flagellum and fine pili-like structures (Fig. 2B). Rarely, cells with two subterminal flagella were also observed. A study by Mayer and Schoberth on the ultrastructure of this organism is being published elsewhere (manuscript in preparation).

**Substrates utilized for growth.** Evidence presented in Table 1 indicates that the acetogenic organism (strain WB1) can oxidize substrates other than hydrogen but that the substrate range is rather narrow, being limited to fructose, glucose, lactate, glycerate, and formate. The only major product detected was acetate;

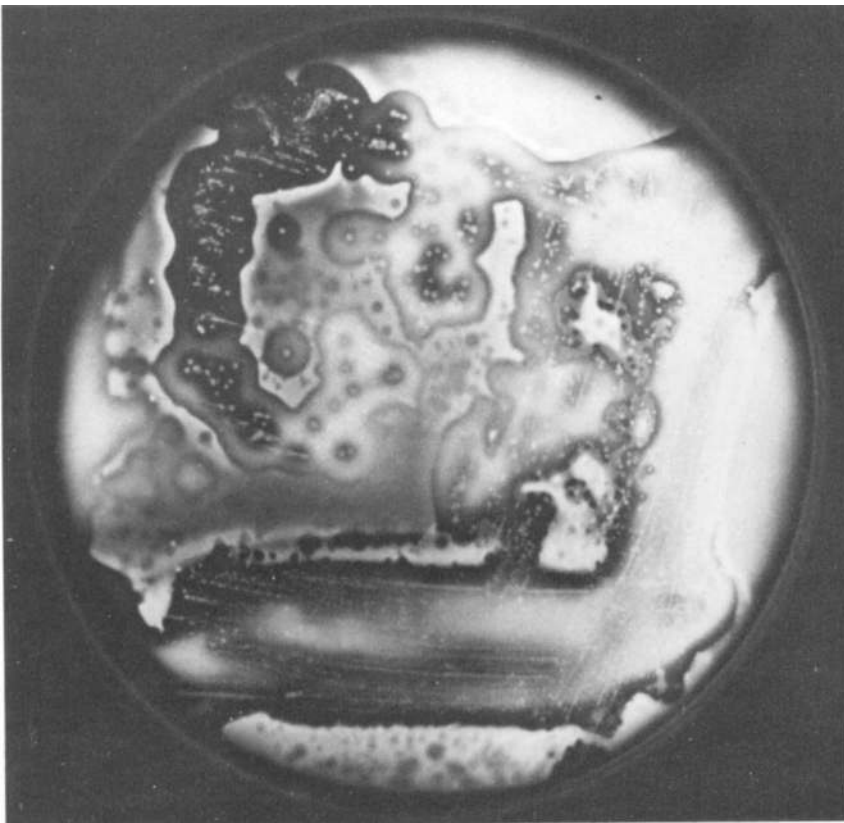


FIG. 1. Colonies of *Acetobacterium woodii* which were grown in an atmosphere of  $H_2$ : $CO_2$  (80:20) on a medium which contained  $CaCO_3$ . Acetogenic colonies are surrounded by clear zones. Magnification,  $\times 1.2$ .

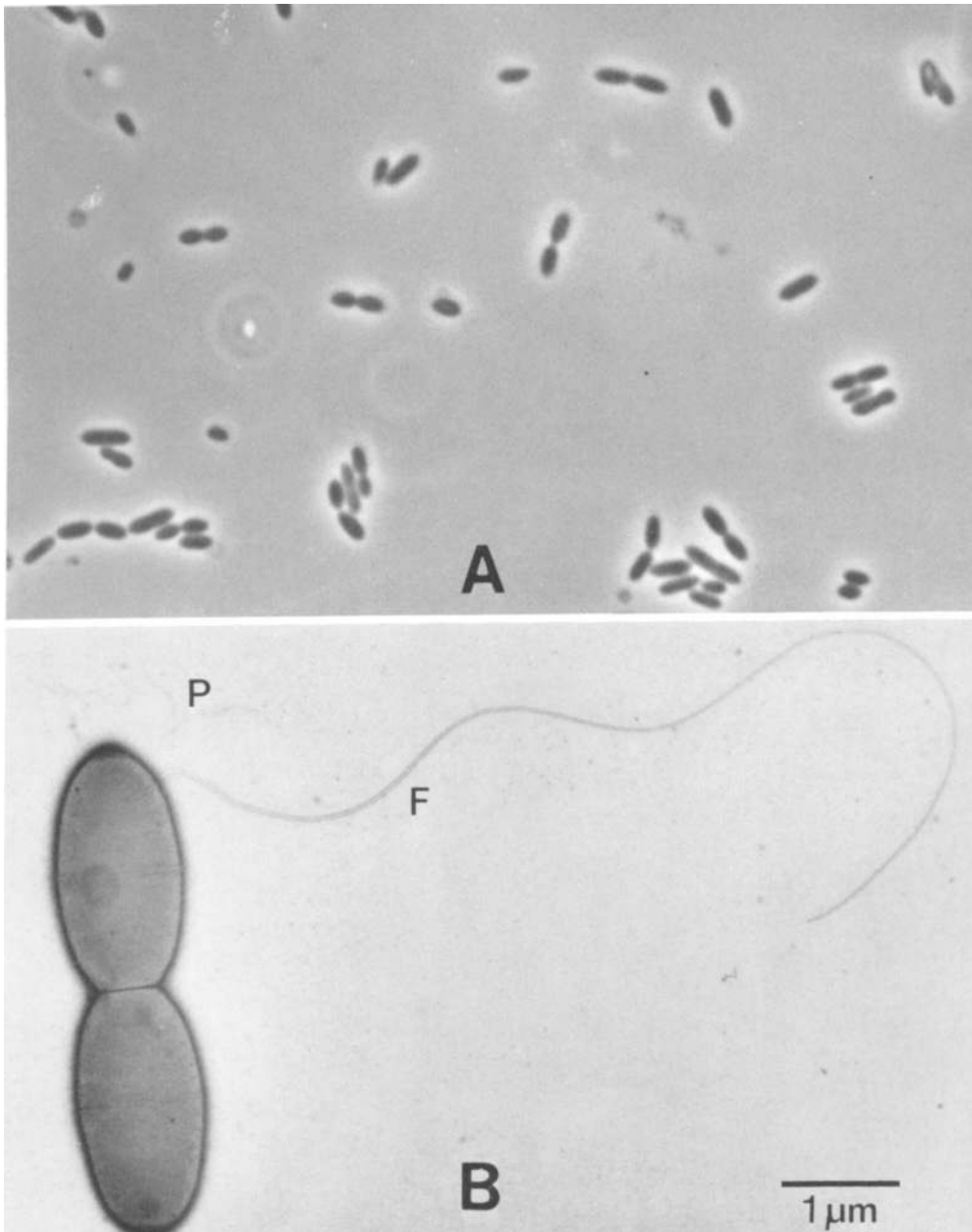


FIG. 2. (A) Phase-contrast photomicrograph of living cells of the acetogenic bacterium. Magnification,  $\times 2,000$ . (B) Electron micrograph of the acetogenic bacterium showing a single, subterminal flagellum, F, and pili-like structures, P. The micrograph was taken by F. Mayer.

traces of a compound which may have been succinate were detected when substrates other than hydrogen were utilized. The results presented in Fig. 3 show that the acetogenic organism produced a homoacetic fermentation: 92

to 95% of the fructose was converted to acetate. The generation time of the organism at  $30^{\circ}\text{C}$  was 6 h. However, no evidence was obtained for the formation of hydrogen during the fermentation. The fermentor was continuously sparged

with nitrogen at a flow rate of 15 cm<sup>3</sup>/min per liter of medium, and the theoretical amount of acetate was nearly formed from the fructose used, indicating that molecular hydrogen likely was not formed since a significant portion would have equilibrated with the medium and would have been scrubbed out with the nitrogen. Growth with H<sub>2</sub>-CO<sub>2</sub> was much slower. In these experiments, the cell yield on the fermented fructose was 2.2 g (wet weight) per liter of medium, and the yield on the gas mixture was 1.5.

**Nutrition.** The results of the nutrition studies (Table 2) indicate that pantothenate alone can replace the vitamin requirement for strain WB1. In this experiment, the growth yield was low; to avoid introducing variables, acetic acid was not neutralized, and the gas atmosphere was not replaced. The organism could be subcultured continually in media which contained calcium pantothenate as the only vitamin.

**Stoichiometry of growth on H<sub>2</sub> and CO<sub>2</sub>.** To determine the amount of hydrogen and carbon dioxide consumed as well as the amount of acetic acid formed by strain WB1, growth was followed in sealed tubes as described in Materials and Methods. Gas consumption and acetate

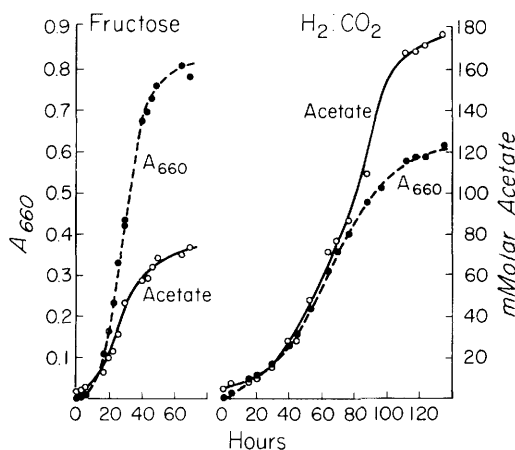


FIG. 3. Growth of and production of acetic acid by the acetogenic bacterium in fermentor cultures. Oxygen-free nitrogen was bubbled (15 cm<sup>3</sup>/min per liter of medium) through the culture in which fructose was the substrate, and an oxygen-free mixture of H<sub>2</sub>:CO<sub>2</sub> (80:20) was bubbled (40 cm<sup>3</sup>/min per liter of medium) through the fermentor from which the data plotted on the right were obtained. Absorbance of samples was determined by use of a 1-cm light path.

TABLE 1. Organic substrates tested for support of growth of the acetogenic bacterium under an atmosphere of nitrogen and carbon dioxide (80:20)

Organic substrate tested	Final absorbance of broth culture <sup>a</sup> (at 660 nm)
Fructose <sup>b</sup>	1.12
Glucose <sup>b</sup>	0.89
DL-Lactate (sodium) <sup>b</sup>	0.50
DL-Glycerate (sodium) <sup>b</sup>	0.35
Formate (sodium) <sup>c</sup>	0.40
Other compounds <sup>d</sup>	<0.10

<sup>a</sup> The inoculated basal medium (without added substrate) supported growth which reached an absorbance <0.1.

<sup>b</sup> This substrate supported the growth of the acetogenic bacterium.

<sup>c</sup> Cells initially grew poorly on formate, but by continual subculture on this substrate, an absorbance of 0.4 was obtained at 100 mM formate.

<sup>d</sup> Compounds tested which did not serve as substrates for the growth of the acetogenic bacterium: methanol, ethanol, *n*-propanol, *i*-propanol, *n*-butanol, *i*-butanol, *sec*-butanol, and *n*-pentanol; acetic, propionic, butyric, valeric, fumaric, L-malic, pyruvic, and succinic acids; alanine, aspartic acid, Casamino Acids, casein hydrolysate, glutamic acid, glycine, and serine; arabinose, cellobiose, cellulose, 2-deoxyglucose, galactose, lactose, mannose, maltose, melezitose, pectin, raffinose, rhamnose, ribose, starch, sucrose, trehalose, xylose, mannitol, inositol, glycerol, alginic acid, galactonolactone, galacturonic acid, gluconolactone, and glucuronic acid.

formation correlated with an increase in the absorbancy of the culture (Fig. 4). During growth, 6 μmol of gas was consumed per μmol of acetate produced. The results of analyses by gas chromatography agreed with equation 1. No growth occurred in tubes where N<sub>2</sub> was substituted for H<sub>2</sub>.

**Additional characteristics of the acetogenic bacterium.** Batches of cells for use in the determination of the G+C content of the DNA were grown on fructose as well as on H<sub>2</sub>-CO<sub>2</sub>. The buoyant density of purified DNA from cells cultured under either condition was 1.699 g/cm<sup>3</sup>, and the calculated G+C content was 39 mol%. To document further the characteristics of the organism, cultures were sent to the Anaerobe Laboratory, Virginia Polytechnic Institute (VPI) and State University, Blacksburg, Va. The results of a series of standard substrate fermentations and reaction tests confirmed our results and indicated that the organism was unlike any of the bacteria in the VPI culture collection (L. V. Holdeman, personal communication).

Additional strains of the acetogenic organism, which are designated RT1 and RT2, also were studied; characteristics of these strains closely resembled those of strain WB1.

## DISCUSSION

Although the acetogenic bacterium is unique in its ability to grow by the oxidation of hydro-

gen and reduction of carbon dioxide to acetic acid, it appears from preliminary studies that the homoacetic fermentation of sugar closely resembles the fermentations carried out by strains of *C. thermoaceticum* and *C. formicoaceticum* (1, 11).

We propose the establishment of a new genus, *Acetobacterium* (A·ce·to·bac·te'rium. L.n. *acetum* vinegar; Gr.neut.n. *bakterion* a small rod; M.L. neut.n. *Acetobacterium* vinegar rod) for these acetogenic bacteria. The type species of this genus is *Acetobacterium woodii* sp. nov. (wood'i·i·M.L. gen.n. *woodii* of Wood, named for Harland G. Wood, who pioneered studies on the total synthesis of acetate from CO<sub>2</sub> by bacteria). Until definitive evidence becomes available, we suggest that *Acetobacterium* be placed in the family *Propionibacteriaceae*. Strains of *Acetobacterium woodii* appear to be most closely related to the genus *Eubacterium*, but they differ in their ability to grow by the anaerobic oxidation of hydrogen and the reduction of CO<sub>2</sub> to acetic acid as well as in their ability to carry out only homoacetic fermentations from reduced substrates. A combined generic and specific description follows.

*Acetobacterium* gen. nov. and *Acetobacterium woodii* sp. nov.

**Morphology.** Oval-shaped, short rods measuring 1.0 by 2.0 μm. Cells frequently occur in pairs. Gram positive. Highly motile by means of one or two subterminal flagella. Endospores are absent.

**Colony characteristics.** Colonies are circular and convex and grow to a diameter of 1 mm

TABLE 2. *Vitamin requirements of the acetogenic bacterium when grown in a mineral medium under a pressurized atmosphere of hydrogen and carbon dioxide (67:33)*

Vitamins added to basal mineral medium	Amount of vitamin added per liter of medium (μg)	Final absorbance of broth culture <sup>a</sup> (at 660 nm)
None		0.04
Biotin	20	0.06
Folic acid	20	0.05
Pyridoxine-hydrochloride	100	0.06
Riboflavin	50	0.04
Ca <sup>2+</sup> pantothenate	50	0.33
Vitamin B <sub>12</sub>	1	0.05
<i>p</i> -Aminobenzoic acid	50	0.05
Thioctic acid	50	0.05
All of the above vitamins plus:		
Thiamine-hydrochloride	50	0.30
Nicotinic acid	50	

<sup>a</sup> Absorbance values, when compared to the blank (0.04), indicate a negligible stimulation of growth by addition of vitamins other than pantothenate.

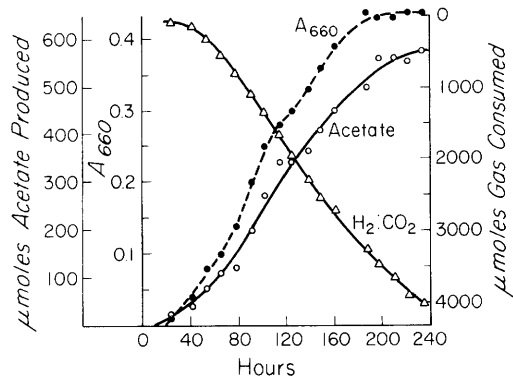


FIG. 4. *Growth and stoichiometry of substrate utilization and acetic acid production by the acetogenic bacterium in a closed, pressurized system.*

in 7 to 10 days. Older colonies may show a slight yellow pigmentation. Traces of a water-soluble yellow pigment may be excreted into the medium. Catalase negative.

**DNA base composition.** 39 mol% G+C.

**Temperature for optimal growth.** 30°C.

**pH relationships.** Remains viable at pH 5 for many weeks.

**Physiology.** Obligately anaerobic. Ferments 1 mol of fructose to 3 mol of acetate; also produces homoacetic fermentations from glucose, lactate, and glycerate. May be adapted to ferment formate. Oxidizes hydrogen and reduces carbon dioxide according to the following equation:  $4\text{H}_2 + 2\text{CO}_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O}$ . Traces of succinate may be produced from organic substrates.

**Nutrition.** Grows in a defined mineral medium with the addition of substrates (H<sub>2</sub>-CO<sub>2</sub> or organic substrate). Pantothenate is required.

**Source.** Marine and fresh-water black sediments.

**Type strain.** WB1. This strain, isolated from a marine estuary, has been deposited in the American Type Culture Collection (ATCC), Rockville, Md., under the number 29683 and in the German Collection of Microorganisms (DSM), Göttingen, FRG, under the number 1030.

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## REPRINT REQUESTS

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