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Acetoanaerobium noterae gen. nov., sp. nov.: an Anaerobic Bacterium That Forms Acetate from H_2 and CO_2

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An anaerobic bacterium which produced acetate from H_2 and CO_2 was isolated. The rod-shaped cells were not lysed by KOH, did not hydrolyze L-alanine-4-nitroanilide, and stained gram negative. However, the cell wall did not resemble a gram-negative wall in structure; it was comprised of two layers. The cells were motile by means of three or four peritrichous flagella. Yeast extract was required for both chemoorganotrophic and chemolithotrophic growth; yeast extract, glucose, maltose, or H_2-CO_2 could serve as a substrate for growth. Strain NOT-3^T (T = type strain) grew best at 37°C and pH 7.6 to 7.8. The deoxyribonucleic acid base composition was 36.8 mol% guanine plus cytosine. Strain NOT-3 (= ATCC 35199) is named *Acetoanaerobium noterae* gen. nov., sp. nov. and is the type strain of this new species.

Acetate production by H_2-dependent CO_2 reduction was first demonstrated in enrichment cultures (9). Subsequently, *Clostridium aceticum*, which grows readily in the presence of H_2 and CO_2, was isolated by Wieringa (20). Acetate is the only product formed by this organism. The original culture of Wieringa was presumably lost for many years but was recently revived from an old endospore preparation (5). Isolates similar to *C. aceticum* have been described by other workers (1, 14). Anaerobic H_2-oxidizing acetogenic bacteria can be found in a number of environments (6). Other species with this property have been described in the genera *Acetobacterium* (2, 4), *Acetogenium* (11), *Eubacterium* (18), and *Clostridium* (19), including thermophilic species (11, 19).

A sediment sample taken from an oil exploration drilling site was examined for the presence of methanogens. High dilutions of the sample showed H_2 uptake and acetate production without methanogenesis. A bacterium which produced acetate from H_2 and CO_2 was isolated from these dilutions. This organism (strain NOT-3^T [= ATCC 35199^T]) (T = type strain) is named *Acetoanaerobium noterae* gen. nov., sp. nov.

(A brief report of this work appeared previously [Sleat, Mah, and Robinson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I54, p. 148].)

MATERIALS AND METHODS

Bacterial strains. Strain NOT-3^T was isolated from sediment of the Notera 3 oil exploration drilling site in the Hula swamp area of Galilee, Israel. The sediment pH was 8.0. Polyethylene bottles were filled with sediment samples, shipped to the laboratory, and stored under O_2-free N_2 at 4°C.

Culture medium. The culture medium used was prepared by using the techniques of Hungate (10). This medium contained (per liter) 0.4 g of $K_2HPO_4 \cdot 3H_2O$, 1.0 g of NH_4Cl, 0.45 g of NaCl, 2.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.15 g of L-cysteine hydrochloride, and 0.001 g of resazurin; 10 ml of a trace metal solution (8) and 10 ml of vitamin solution (5) were added per liter. The medium was adjusted to pH 7.0 with 4 N NaOH, dispensed under a gas phase of either H_2-CO_2 (4:1) or N_2-CO_2 (4:1), and autoclaved. Before inoculation the pH was adjusted (usually to 8.0) with a sterile Na_2CO_3 solution, and $Na_2S \cdot 9H_2O$ (final concentration, 0.15 g/liter) was added from a sterile stock solution. Solid media also contained 1.5% purified agar (Difco). For substrate utilization experiments the yeast extract concentration was reduced to 0.5 g/liter, and substrate was added from a sterile anaerobic stock solution to give a final concentration of 10 mM.

Most-probable-number determinations. The medium used for most-probable-number determinations contained (per liter) 2.0 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 2.5 g of sodium acetate, and 2.5 g of sodium formate. Sediment (1 g, wet weight) was added to 5 ml of medium, the container was aseptically stoppered, and the preparation was blended vigorously in a Vortex mixer. The resulting sediment slurry was decimally diluted; 0.2 ml of each dilution was inoculated into each of five tubes containing 5 ml of medium with an H_2-CO_2 gas phase. The tubes were incubated at 35°C with vigorous shaking. H_2 utilization and CH_4 production were determined by gas analysis (3). The most probable numbers of H_2-oxidizing bacteria were determined from previously published statistical tables (13).

Isolation of DNA and analysis of base composition. Deoxyribonucleic acid (DNA) was extracted with chloroform-isoamyl alcohol and purified (16). The buoyant density of the purified DNA was determined by ultracentrifugation is a CsCl gradient (15). DNAs from *Escherichia coli* strain B and *Clostridium perfringens* were used as references. The DNA guanine-plus-cytosine content was calculated from its buoyant density (17).

Microscopy. A Zeiss Universal Research microscope (Carl Zeiss, Oberkochen, West Germany) equipped with phase optics was used for photomicroscopy. For thin-section electron microscopy, broth-grown cells were fixed for 20 min in 0.1 M cacodylate buffer (pH 7.2) containing 2% formaldehyde and 2.5% glutaraldehyde. After the cells were washed with buffer, they were fixed in buffered 1% OsO_4 for 1 h. The cells were then enrobed in agar, dehydrated, and embedded in Spurr plastic. Thin sections were stained with uranyl and lead salts. Sections were examined with a Jeol model 100CX electron microscope. For scanning electron microscopy, a colony growing on agar was fixed for 20 min with 2.5% glutaraldehyde in cacodylate buffer. It was then dehydrated in alcohol, critical-point dried, and gold coated. The colony

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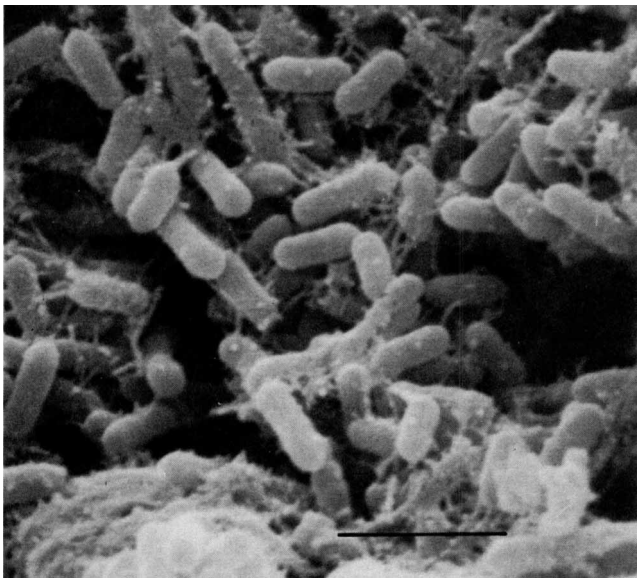

FIG. 1. Scanning electron photomicrograph of cells of *Acetoanaerobium noterae* from a roll tube colony. Bar = 1 μm.

was observed with a Hitachi model S-450 scanning electron microscope.

Analytical techniques. Gram stain reactions, lysis by KOH, and hydrolysis of L-alanine-4-nitroanilide were determined by the methods of Carlone et al. (7). Growth of strain NOT-3^T in broth was measured by absorbance at 540 nm. Gases were analyzed by gas chromatography, and volatile fatty acids were measured by gas chromatography after acidification with H_3PO_4 and centrifugation (3).

RESULTS AND DISCUSSION

Numbers of H_2-oxidizing acetogens. The most-probable-number analysis of the drilling site sample yielded 1.75×10^5 H_2-oxidizing acetogens per g (wet weight). No methane was detected in any of the tubes. A microscopic examination of the cultures positive for H_2 utilization revealed bacteria with morphology similar to that of the isolate, strain NOT-3^T; using tubes from the highest dilution as inoculum, we isolated several H_2-oxidizing acetogens morphologically similar to strain NOT-3^T.

Isolation of strain NOT-3^T. Non-methanogenic H_2-oxidizing organisms were enriched by inoculating 0.5 g of sediment into 5 ml of medium with H_2-CO_2 gas; this enrichment culture was incubated at 35°C for 3 days. No CH_4 was detected, but H_2 uptake occurred. Decimal dilutions of the enrichment were made, and roll tubes were inoculated at each dilution. After incubation for 24 h, punctiform colonies appeared at the high dilution. Several of these colonies were picked, diluted, and inoculated into roll tube media for purification. One typical rod-shaped isolate, strain NOT-3^T (Fig. 1), was chosen for further study; it was picked, transferred to liquid medium, and subsequently maintained by 5% transfer every 14 days.

The cells stained gram negative, did not hydrolyze L-alanine-4-nitroanilide, and were not lysed by KOH. However, the double-layered cell wall did not resemble a typical

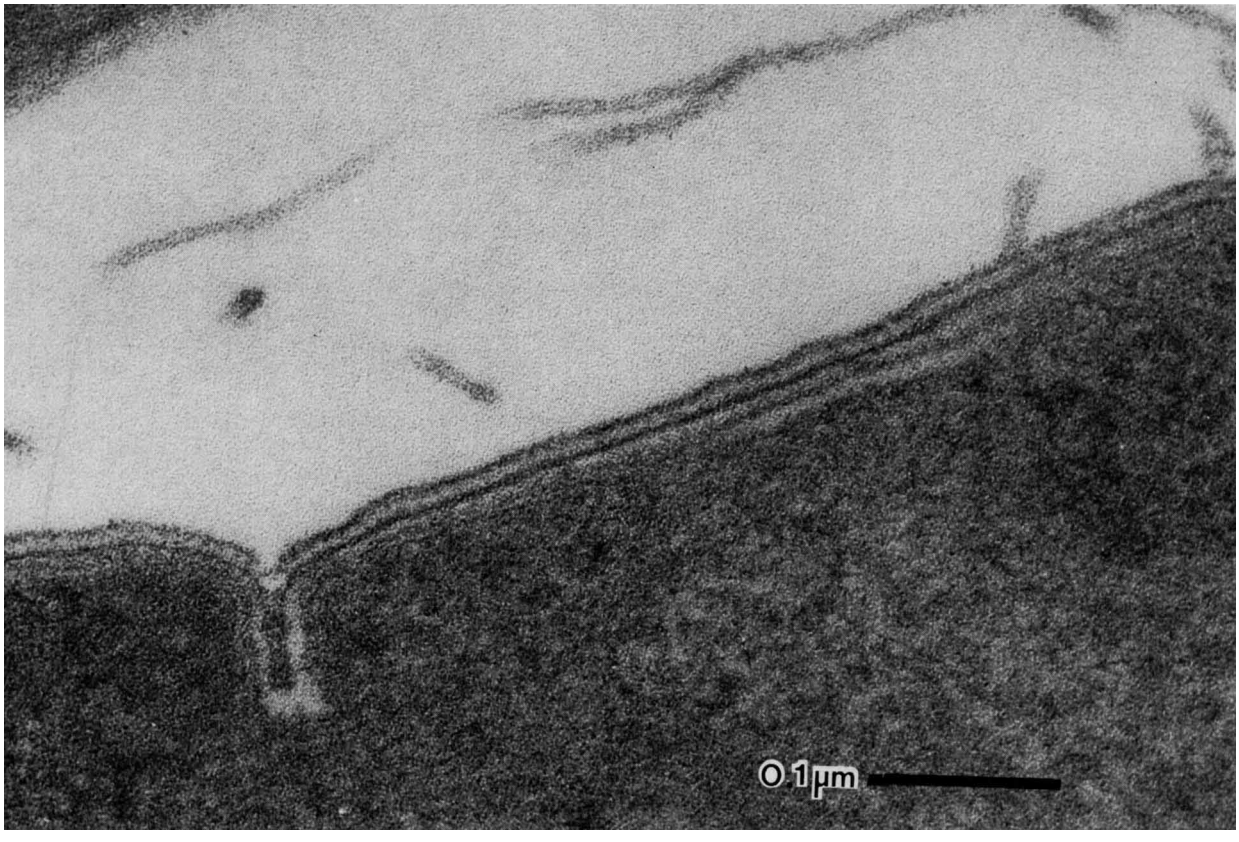

FIG. 2. Thin section of *Acetoanaerobium noterae* showing two-layer cell wall.

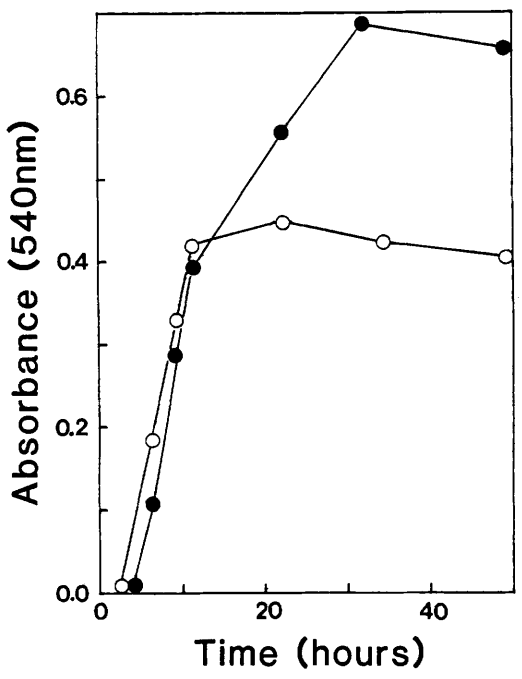

FIG. 3. Growth of *Acetoanaerobium noterae* on yeast extract with an N_2-CO_2 (●) or H_2-CO_2 (○) gas phase.

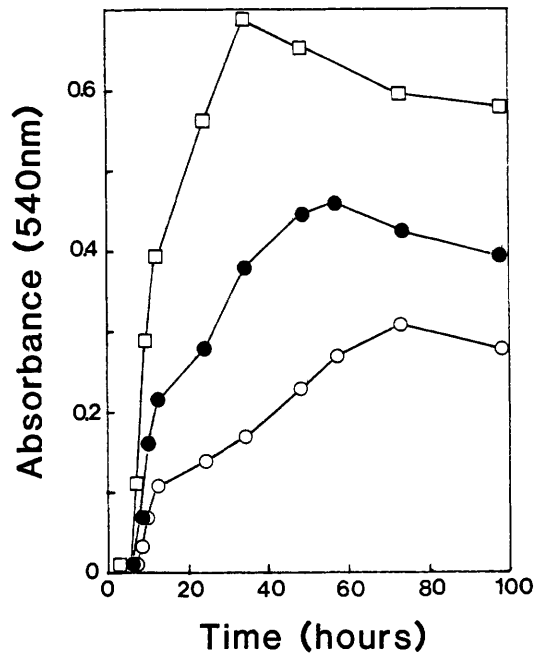

FIG. 4. Growth of *Acetoanaerobium noterae* with H_2-CO_2 and the following concentrations of yeast extract: 0.05% (wt/vol) (○), 0.1% (wt/vol) (●), and 0.2% (wt/vol) (□).

gram-negative cell wall in structure (Fig. 2). In liquid culture, cells often occurred in pairs and, during the stationary phase, in long chains. Endospores were not found by either phase-contrast or electron microscopy. Spore staining of old cultures by the malachite green method also failed to reveal the presence of endospores. Furthermore, no growth occurred after cultures were exposed to 70°C for 15 min.

Higher cell yields were obtained on yeast extract medium when H_2-CO_2 replaced N_2-CO_2 as the gas phase. In fact, growth was biphasic (Fig. 3) under these conditions. The initial rapid growth phase was similar to growth in the absence of H_2. There followed a period of growth at a greatly reduced rate, which occurred only in the presence of H_2. H_2 uptake occurred throughout both phases of the growth cycle but was greatly increased during the second phase of growth (H_2-dependent growth). Some cultures utilized H_2 for more than 100 days after the onset of the stationary growth phase. Higher initial concentrations of yeast extract increased the rate of H_2-dependent growth, and higher cell yields were obtained (Fig. 4). H_2 uptake occurred over a much longer time at the lower initial yeast extract concentrations. However, in the complete absence of yeast extract, H_2 was not utilized.

In the presence of H_2-CO_2, acetate production was stoichiometrically and temporally related to H_2 uptake during the H_2-utilizing phase of growth (Table 1 and Fig. 5). The theoretical stoichiometry yielded 1 mol of acetate per 4 mol of H_2 used; we found approximately 4.3 mol of H_2 consumed per mol of acetate formed. In addition to an increase in acetate production, a much smaller increase in butyrate production (Table 1) also occurred during the H_2-dependent growth phase. When either glucose or maltose served as the substrate, acetate was the only fermentation product. However, when yeast extract served as the substrate, propionate, butyrate, isobutyrate, and isovalerate were also formed (Table 1).

Taxonomy. Strain NOT-3^T differed significantly from previously described bacteria which produce acetate from H_2 and CO_2 (Table 2). The acetogenic organisms selected for comparison in Table 2 have guanine-plus-cytosine contents ranging from 33 to 43 mol%. The value for strain NOT-3^T fell within this range. Data for *Eubacterium limosum* (18) and *Clostridium thermoautotrophicum* (19) were omitted from Table 2 because the guanine-plus-cytosine contents of these organisms (49 and 53 to 55 mol%, respectively) fall outside this range, and thus strain NOT-3^T was not closely related to these species.

Strain NOT-3^T is not a member of the genus *Clostridium* because it did not form endospores. It is not a member of the genus *Acetobacterium* because it stained gram negative. It differed from *Acetogenium kivui* in cell wall structure and temperature optimum, as well as several other characteristics (11, 12); these differences warrant recognition of strain NOT-3^T as a new species representing a new genus. Thus, we name strain NOT-3^T *Acetoanaerobium noterae* gen. nov., sp. nov.

We propose that the genus *Acetoanaerobium* be placed in the family *Bacteroidaceae* with the generic description given below.

TABLE 1. Volatile fatty acids produced by *Acetoanaerobium noterae* during growth on yeast extract[a]

Gas phase	H_2 uptake (μmol)	Volatile fatty acids formed (μmol)[b]				
		Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate
80% N_2–20% CO_2		154	21	10	15	17
80% H_2–20% CO_2	2,193	643	23	9	23	16

[a] The experimental vials contained N1 medium supplemented with 0.2% yeast extract and were inoculated with 0.1 ml of a culture grown on 0.2% yeast extract under H_2–CO_2. The vials were incubated at 37°C with shaking for 376 h.

[b] Values are averages of three experimental vials.

***Acetoanaerobium* gen. nov.** *Acetoanaerobium* (A.ce.to.an.ae. ro'bi.um. L. n. *acetum* vinegar; Gr. pref. *an* not; Gr. n. *aer* air; Gr. n. *bios* life; M. L. neut. n. *Acetoanaerobium* vinegar anaerobe) cells are nonsporeforming rods. Cells stain gram negative but have an atypical gram-negative wall structure.

Chemoorganotrophs. Ferment carbohydrates, producing acetate and sometimes other volatile acids. Ferment yeast extract, producing acetate and several volatile acids. At slower growth rates produce acetate from H_2 and CO_2; may require yeast extract for growth.

Obligately anaerobic.

The type species is *Acetoanaerobium noterae*.

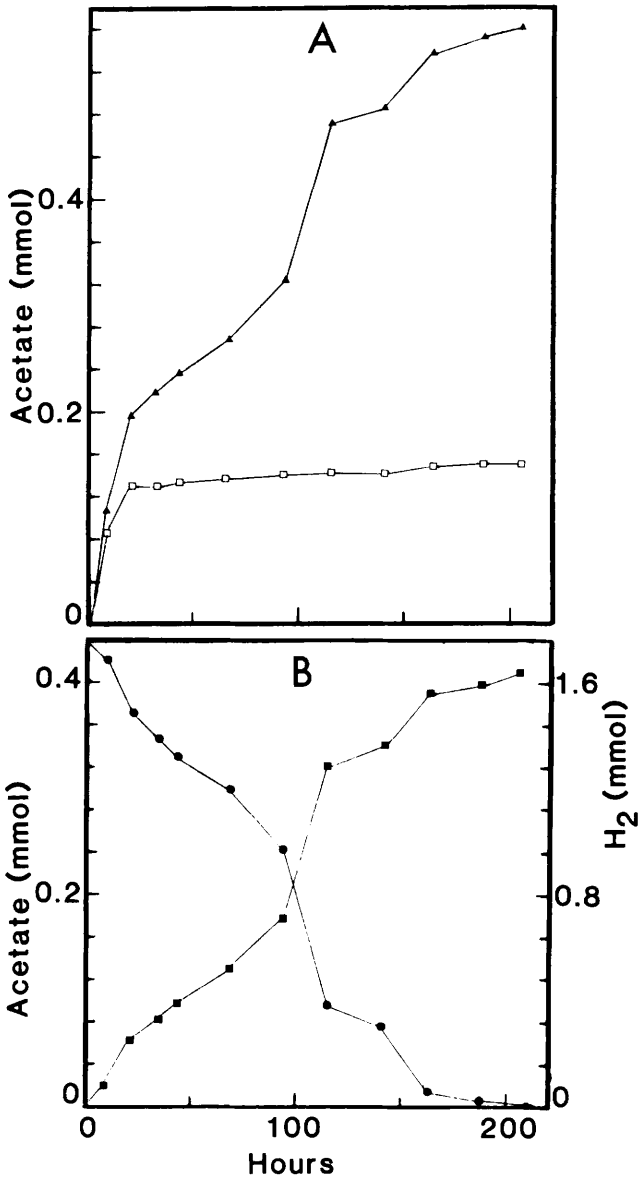

FIG. 5. H_2-dependent acetate formation from H_2-CO_2 in the presence of 0.2% (wt/vol) yeast extract. (A) Acetate formation in the presence of N_2-CO_2 and 0.2% (wt/vol) yeast extract (□) or H_2-CO_2 and 0.2% yeast extract (▲). (B) Net acetate formation (total acetate production minus acetate production from yeast extract alone) (■) verus H_2 utilization (●).

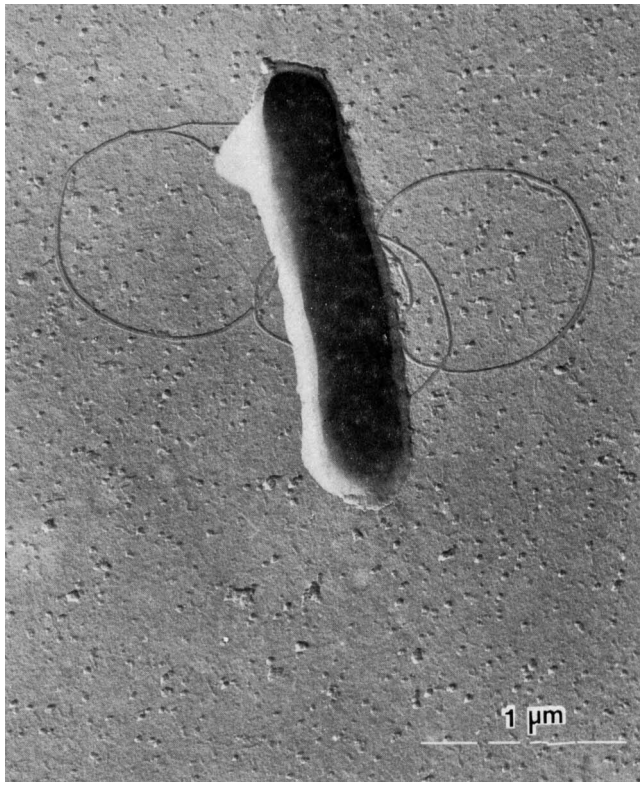

FIG. 6. Platinum shadow replica of *Acetoanaerobium noterae* showing three or four peritrichous flagella.

***Acetoanaerobium noterae* sp. nov.** *Acetoanaerobium noterae* (no'ter.ae. L. adj. *noterae* pertaining to Notera; named for its source, the Notera oil exploration site in Israel) cell are straight rods that are 0.8 μm wide and 1 to 5 μm long. Motile, with three or four peritrichous flagella (Fig. 6). Cells stain gram negative; the cell wall is atypical and, as determined by transmission electron microscopy, is composed of two distinct layers, a darker inner layer and lighter outer layer. Colonies are rhizoid, opaque, and granular (Fig. 7). Young colonies are white, but older colonies are brownish and up to 2 cm in diameter after 1 month of incubation.

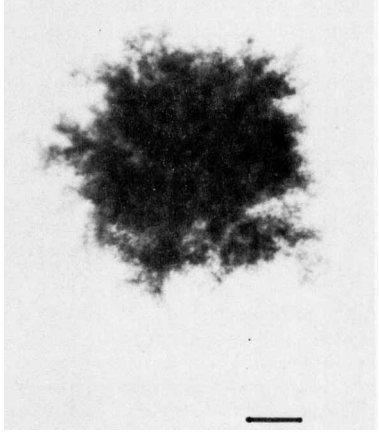

FIG. 7. Photomicrograph of an *Acetoanaerobium noterae* colony. Bar = 1 mm.

TABLE 2. Comparison of stain NOT-3T with other H_2-oxidizing acetogenic bacteria

Characteristic	*C. aceticum* DSM 1496	*Acetobacterium woodii* ATCC 29683	*Acetobacterium weiringae* DSM 1911	*Acetogenium kivui* ATCC 33488	*Acetoanaerobium noterae* ATCC 35199^T
Cell morphology	Rods	Oval rods	Oval rods	Rods	Rods
Cell width (μm)	0.8–1.0	1	1	0.7–0.8	0.8
Cell length (μm)	5	2	1–2	2–7.5	1–5
Gram reaction	−	+	+	−[a]	−[a]
Motility	+	+	+	−	+
Flagella	Peritrichous	Subterminal	Subterminal	None	Peritrichous
Spore formation	+	−	−	−	−
Colony form	Rhizoid	Circular	Circular	Circular	Rhizoid
pH optimum	8.3	NR[b]	7.2–7.8	6.4	7.6
Temp optimum (°C)	30	30	30	66	37
Substrates fermented					
Fructose	+	+	+	+	−
Glucose	−	+	−	+	+
Maltose	−	−	−	−	+
Pyruvate	+	−	−	+	−
Yeast extract required for H_2 oxidation	+	−	−	−	+
Guanine-plus-cytosine content (mol %)	33	39	43	38	37
Reference	5	2	4	11	

[a] Transmission electron micrographs show gram-positive cell wall structure of *Acetogenium kivui* but an atypical double-layered wall of strain NOT-3^T.
[b] NR, Not reported.

Yeast extract, maltose, and glucose are used for heterotrophic growth. Compounds not supporting growth include arabinose, rhamnose, ribose, xylose, fructose, galactose, cellobiose, lactose, mannose, sucrose, melezitose, trehalose, erythritol, adonitol, arabitol, dulcitol, inositol, mannitol, sorbitol, formate, acetate, pyruvate, lactate, malate, fumarate, succinate, citrate, glutamate, methylamine, trimethylamine, and methanol.

Yeast extract is required for growth and H_2 utilization. Growth on yeast extract and H_2-CO_2 is biphasic, with an initial rapid growth phase independent of the presence of H_2. This is followed by H_2-dependent acetate production during the second slower growth phase.

Cells produce acetate, propionate, isobutyrate, butyrate, and isovalerate (and little or no H_2) during growth on yeast extract alone. When either glucose or maltose serves as a substrate, acetate is the only fermentation product.

Obligately anaerobic. Growth is most rapid at 37°C and pH 7.6 (pH range, 6.6 to 8.4). Minimum doubling times are 2.8 h for heterotrophic growth and 27 h for H_2-dependent growth. Vitamins are not required. Growth is inhibited by erythromycin, chloramphenicol, penicillin, cephalosporin, and cycloserine at concentrations of 100 ng/liter.

The DNA base composition is 37 mol% guanine plus cytosine.

Isolated from sediment near the Notera oil drilling site in the Hula swamp area of Galilee, Israel.

The type strain is strain NOT-3 (= ATCC 35199).

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