Acetoanaerobium noterae gen. nov., sp. nov.: an Anaerobic Bacterium That Forms Acetate from H\textsubscript{2} and CO\textsubscript{2}

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An anaerobic bacterium which produced acetate from H\textsubscript{2} and CO\textsubscript{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 chemolithothrophic growth; yeast extract, glucose, maltose, or H\textsubscript{2}-CO\textsubscript{2} could serve as a substrate for growth. Strain NOT-3\textsuperscript{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\textsubscript{2}-dependent CO\textsubscript{2} reduction was first demonstrated in enrichment cultures (9). Subsequently, Clostridium aceticum, which grows readily in the presence of H\textsubscript{2} and CO\textsubscript{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\textsubscript{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), Acetogentium (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\textsubscript{2} uptake and acetate production without methanogenesis. A bacterium which produced acetate from H\textsubscript{2} and CO\textsubscript{2} was isolated from these dilutions. This organism (strain NOT-3\textsuperscript{T} [= ATCC 35199\textsuperscript{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, 154, p. 148].)

MATERIALS AND METHODS

**Bacterial strains.** Strain NOT-3\textsuperscript{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\textsubscript{2}-free N\textsubscript{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 KH\textsubscript{2}PO\textsubscript{4} - 3H\textsubscript{2}O, 1.0 g of NH\textsubscript{4}Cl, 0.45 g of NaCl, 2.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.15 g of D-cysteine hydrochloride, and 0.001 g of resazurin; 10 ml of a trace metal solution (8) and 10 ml of a vitamin solution (5) were added per liter. The pH was adjusted to pH 7.0 with 4 N NaOH, dispensed under a gas phase of either H\textsubscript{2}-CO\textsubscript{2} (4:1) or N\textsubscript{2}-CO\textsubscript{2} (4:1), and autoclaved. Before inoculation the pH was adjusted (usually to 8.0) with a sterile Na\textsubscript{2}CO\textsubscript{3} solution, and Na\textsubscript{2}S - 9H\textsubscript{2}O (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\textsubscript{2}-CO\textsubscript{2} gas phase. The tubes were incubated at 35°C with vigorous shaking. H\textsubscript{2} utilization and CH\textsubscript{4} production were determined by gas analysis (3). The most probable numbers of H\textsubscript{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-isomyl alcohol and purified (16). The buoyant density of the purified DNA was determined by ultracentrifugation in 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% Os\textsubscript{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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RESULTS AND DISCUSSION

Numbers of H₂-oxidizing acetogens. The most-probable-number analysis of the drilling site sample yielded $1.75 \times 10^5$ H₂-oxidizing acetogens per g (wet weight). No methane was detected in any of the tubes. A microscopic examination of the cultures positive for H₂ utilization revealed bacteria with morphology similar to that of the isolate, strain NOT-3ᵀ; using tubes from the highest dilution as inoculum, we isolated several H₂-oxidizing acetogens morphologically similar to strain NOT-3ᵀ.

Isolation of strain NOT-3ᵀ. Non-methanogenic H₂-oxidizing organisms were enriched by inoculating 0.5 g of sediment into 5 ml of medium with H₂-CO₂ gas; this enrichment culture was incubated at 35°C for 3 days. No CH₄ was detected, but H₂ 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-3ᵀ (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
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 H₂-CO₂ replaced N₂-CO₂ 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 H₂. There followed a period of growth at a greatly reduced rate, which occurred only in the presence of H₂. H₂ uptake occurred throughout both phases of the growth cycle but was greatly increased during the second phase of growth (H₂-dependent growth). Some cultures utilized H₂ for more than 100 days after the onset of the stationary growth phase. Higher initial concentrations of yeast extract increased the rate of H₂-dependent growth, and higher cell yields were obtained (Fig. 4). H₂ uptake occurred over a much longer time at the lower initial yeast extract concentrations. However, in the complete absence of yeast extract, H₂ was not utilized.

In the presence of H₂-CO₂, acetate production was stoichiometrically and temporally related to H₂ uptake during the H₂-utilizing phase of growth (Table 1 and Fig. 5). The theoretical stoichiometry yielded 1 mol of acetate per 4 mol of H₂ used; we found approximately 4.3 mol of H₂ 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 H₂-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-3T differed significantly from previously described bacteria which produce acetate from H₂ and CO₂ (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-3T 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-3T was not closely related to these species.

Strain NOT-3T 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 kivai in cell wall structure and temperature optimum, as well as several other characteristics (11, 12); these differences warrant recognition of strain NOT-3T as a new species representing a new genus. Thus, we name strain NOT-3T Acetoanaerobium noterue 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 noterue during growth on yeast extract

<table>
<thead>
<tr>
<th>Gas phase</th>
<th>H₂ uptake (μmol)</th>
<th>Acetate Propionate Isobutyrate Butyrate Isovalerate</th>
</tr>
</thead>
<tbody>
<tr>
<td>80% N₂:20% CO₂</td>
<td>154 21 10 15 17</td>
<td></td>
</tr>
<tr>
<td>80% H₂:20% CO₂</td>
<td>2,193 643 23 9 23 16</td>
<td></td>
</tr>
</tbody>
</table>

*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 H₂-CO₂. The vials were incubated at 37°C with shaking for 37h.

*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. Acetoan-
aerobium 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 H₂ and CO₂; may
require yeast extract for growth.

Obligately anaerobic.
The type species is *Acetoanaerobium noterae*.

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**Acetoanaerobium noterae** sp. nov. Acetoanaerobium not-
erae (no'ter.ae. L. adj. noter.ae 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 deter-
mined 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.

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**Fig. 5.** H₂-dependent acetate formation from H₂-CO₂ in the
presence of 0.2% (wt/vol) yeast extract. (A) Acetate formation in the
presence of N₂-CO₂ and 0.2% (wt/vol) yeast extract (●) or H₂-CO₂,
and 0.2% yeast extract (▲). (B) Net acetate formation (total acetate
production minus acetate production from yeast extract alone) (●)
versus H₂ utilization (○).

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

**Fig. 7.** Photomicrograph of an *Acetoanaerobium noterae*
colony. Bar = 1 mm.
Yeast extract, maltose, and glucose are used for heterotrophic growth. Compounds not supporting growth include arabinose, rhamnose, xylose, fructose, galactose, cellobiose, lactose, mannose, sucrose, melezitose, trehalose, erythritol, adonitol, arabitol, dulcitol, inositol, manitol, sorbitol, formate, acetate, pyruvate, lactate, malate, fumarate, succinate, citrate, glutamate, methionine, trimethylamine, and methanol.

Yeast extract is required for growth and H2 utilization. Growth on yeast extract and H2-CO2 is biphasic, with an initial rapid growth phase independent of the presence of H2. This is followed by H2-dependent acetate production during the second slower growth phase.

Cells produce acetate, propionate, isobutyrate, butyrate, and isovalerate (and little or no H2) 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 is inhibited by vitamins. Growth is not inhibited by erythromycin, chloramphenicol, penicillin, cephalosporin, and cycloserine at concentrations of 100 ng/ml.

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

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

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LITERATURE CITED