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Oxidation of primary aliphatic alcohols by *Acetobacterium carbinolicum* sp. nov., a homoacetogenic anaerobe

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Abstract. Four strains of new homoacetogenic bacteria were enriched and isolated from freshwater sediments and sludge with ethanol, propanol, 1,2-propanediol, or 1,2-butanediol as substrates. All strains were Gram-positive nonsporeforming rods and grew well in carbonate-buffered defined media under obligately anaerobic conditions. Optimal growth occurred at 27° C around pH 7.0. H_2/CO_2, primary aliphatic alcohols C_1-C_5, glucose, fructose, lactate, pyruvate, ethylene glycol, 1,2-propanediol, 2,3-butanediol, acetoin, glycerol, and methyl groups of methoxylated benzoate derivates and betaine were fermented to acetate or, in case of primary alcohols C_3-C_5 and 1,2-propanediol, to acetate and the respective fatty acid. In coculture with methanogens methane was formed, probably due to interspecies hydrogen transfer. Strain WoProp1 is described as a new species, *Acetobacterium carbinolicum*. It had a DNA base composition of $38.5 \pm 1.0\%$ guanine plus cytosine, and contained murein of crosslinkage type B similar to *A. woodii*.

Key words: *Acetobacterium carbinolicum* species description – Homoacetogenic fermentations – Incomplete oxidation – Anaerobic degradation – Alcohol oxidation – Diols – Syntrophy – Interspecies hydrogen transfer

Primary aliphatic alcohols are formed during anaerobic degradation of organic matter. A variety of facultatively and obligately anaerobic bacteria ferments carbohydrates to form ethanol, propanol, and butanol among other fermentation products. Under anaerobic conditions, alcohols are oxidized to the respective fatty acid with concomitant reduction of external electron acceptors, e.g. sulfate (Postgate and Campbell 1966). In the absence of external electron acceptors, electrons derived from alcohol oxidation can be released as molecular hydrogen which is used by hydrogen-oxidizing bacteria, e.g. methanogenic bacteria. *Methanobacillus omelianskii* is such a syntrophic association of two bacteria which converts ethanol to acetate and methane (Barker 1941; Bryant et al. 1967).

A third way of degradation of primary alcohols is the fermentation to fatty acids as reduced end products. *Clostridium kluyveri* ferments ethanol with acetate to butyrate (Bornstein and Barker 1948). The sulfate-reducing bacterium *Desulfobulbus propionicus* ferments ethanol in the absence of sulfate to acetate and propionate using carbon dioxide as electron acceptor (Laanbroek et al. 1982). A similar fermentation pattern was reported for an unidentified anaerobic bacterium (Samain et al. 1982) and for *Pelobacter propionicus* (Schink 1984a).

Clostridium aceticum oxidizes ethanol to acetate and uses carbon dioxide as electron acceptor to form further acetate (Wieringa 1940; Braun et al. 1981), according to the equation:

$$2CH_3CH_2OH + 2HCO_3^- \rightarrow 3CH_3COO^- + H^+ + 2H_2O.$$

In the present study, a new anaerobic bacterium physiologically similar to *C. aceticum* is described which ferments primary aliphatic alcohols and bicarbonate to the respective fatty acids and acetate. Since the new isolate is cytologically similar to *Acetobacterium woodii* (Balch et al. 1977), however, differs from this species by its ability to oxidize primary aliphatic alcohols, it is described as a new species, *A. carbinolicum*.

Materials and methods

Sources of organisms

Four strains were enriched and isolated in pure culture from freshwater mud samples:

Strain WoProp1 and strain AS1.2Bd1 from black anoxic mud of a creek and a ditch, respectively, near Konstanz, FRG.

Strain Gö1.2Pd1 from anoxic digestor sludge of municipal sewage plants at Göttingen, FRG.

Strain OttEtOH1 from black anoxic mud of a creek near Hannover, FRG.

Methanospirillum hungatei M 1 h was isolated from digested sludge of the sewage plant at Göttingen, FRG, and cultivated on freshwater medium with 10 mmol/l acetate under H_2/CO_2 mixture (80%/20%).

Acetobacterium woodii strain NZva16 was provided by A. Tschech, University of Konstanz.

Media and growth conditions

All procedures for cultivation and isolation as well as all methods for analysis of metabolic products were exactly as described in earlier papers (Widdel and Pfennig 1981; Schink and Pfennig 1982; Schink 1984a). The mineral medium for enrichment and further cultivation contained 30 mM

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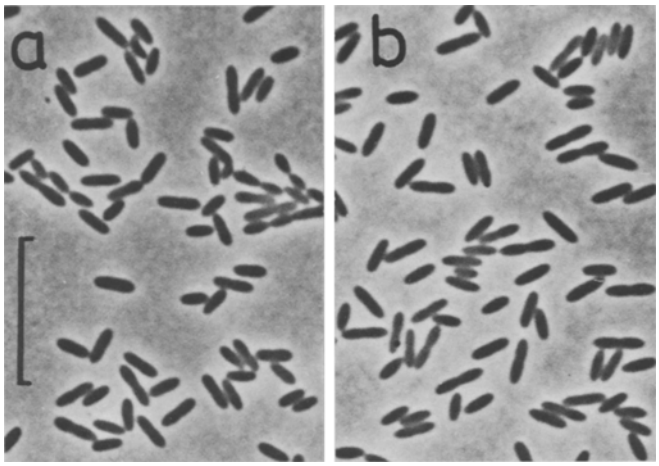

Fig. 1 a, b. Phase-contrast photomicrographs of *Acetobacterium carbinolicum*. **a** Strain WoProp1 enriched with propanol. **b** Strain OttEtOH1 enriched with ethanol. Bar = 10 µm

sodium bicarbonate as buffer, sodium sulfide as reducing agent, and the new trace element solution SL9 (Tschech and Pfennig 1984). The pH was 7.0–7.2. Growth experiments were carried out at 28° C. For isolation of pure cultures, the agar shake culture method (Pfennig 1978) was applied. All chemicals used were of reagent grade quality.

Results

Enrichment and isolation

Enrichment cultures with freshwater medium (50 ml) containing 10 mmol/l of either ethanol, propanol, 1,2-propanediol, or 1,2-butanediol as substrates were inoculated with 5 ml freshwater mud samples from different sources. Methane production was observed after 1–2 weeks of incubation. In subcultures, gas production was not detectable any more after the fourth transfer. Isolation of pure cultures was carried out in agar shake series using the same substrate as in the enrichment cultures. Bacteria formed white to yellowish lens-shaped colonies in the agar. Colonies were resuspended in mineral medium and again purified in agar shake series. Purity was checked by microscopical control and by growth tests in AC medium in which only the isolated cell types could be observed. Finally four strains were chosen for further characterization.

Cytological properties

All strains isolated were rods, 0.8–1.3 × 1.8–3.5 µm in size, with slightly pointed ends. Cells tended to form chains of two or three cells (Fig. 1). Cells of all strains were motile, but lost motility in ageing cultures. All strains stained Gram-positive. Electron microscopic examination of ultrathin sections of two strains showed typical Gram-positive cell wall structures (Fig. 2). The murein chemistry was kindly examined by Prof. Dr. Otto Kandler, München. He detected a B-type murein very similar to that of *Acetobacterium woodii* (Kandler and Schoberth 1979) with a mol ratio of ornithine:glutamate:serine:alanine of 2:1:1:0.8. A small part of ornithine was replaced by lysine. None of our strains formed spores neither in defined medium nor in special

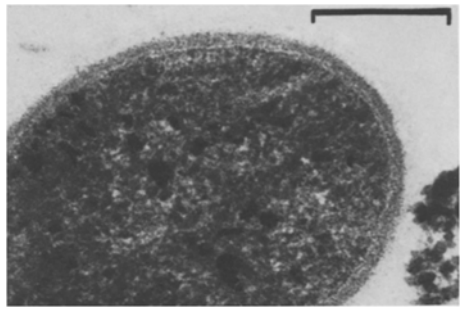

Fig. 2. Electron micrograph of ultrathin section of *Acetobacterium carbinolicum* strain WoProp1. Bar = 0.2 µm

sporulation medium (Hollaus and Sleytr 1972). Enrichment cultures with ethanol and pasteurized mud samples from four sources did not show any growth after 8 weeks of incubation. The guanine plus cytosine content of the DNA was determined with two strains and *Escherichia coli* strain K12 (DSM 498) as reference. It was 38.5 ± 1.0 mol% with strain WoProp1 and 41.3 ± 1.0 mol% with strain OttEtOH1.

No cytochromes could be detected in redox difference spectra of crude cell extracts or membrane preparations.

Physiology

All strains grew well in freshwater medium containing up to 0.72% (w/v) NaCl and 0.15% (w/v) $MgCl_2 \cdot 6H_2O$; no growth occurred in saltwater medium [2.0% (w/v) NaCl, 0.3% (w/v) $MgCl_2 \cdot 6H_2O$]. Phosphate inhibited growth slightly at ≥ 50 mmol/l concentration. The strains grew well in defined medium with vitamin and trace element solutions. Yeast extract was not required for growth, but all strains grew faster and to higher cell densities in the presence of 0.05% yeast extract.

Methanol, ethanol, propanol, butanol, pentanol, several diols, acetoin, glycerol, malate, pyruvate, glucose, and fructose were used as substrates. Autotrophic growth was found on formate and hydrogen/carbon dioxide. Methoxylated derivates of benzoic acid were demethylated to the respective phenols. Neither cinnamic acid nor caffeic acid was reduced to phenylpropionic acids. Neither nitrate, sulfate, thiosulfate, nor sulfur was reduced during degradation of primary alcohols. The results of all substrate tests are summarized in Table 1. Degradation products of primary alcohols and 1,2-diols were the corresponding fatty acids and acetate. With all other substrates, acetate was the only fermentation product.

The growth curve (Fig. 3) illustrates the correlation of growth, ethanol decomposition, and acetate formation by strain WoProp1. Optimal growth (μ = 0.087 h^{-1}; t_d = 8.0 h) occurred at 27° C, the temperature limits were 15° C and 40° C. The pH-optimum was at pH 7.0–7.2; the pH limits were pH 6.0 and 8.0.

Growth yields and stoichiometry

The stoichiometry of substrate utilization and product formation was measured with all strains. The results obtained with strain WoProp1 are presented in Table 2. The amounts of products formed agreed with complete conver-

sion of the substrates to acetate and other fatty acids according to the following equations:

Hydrogen/carbon dioxide:

$$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$$

Formate:

$$4HCOO^- + H^+ \rightarrow CH_3COO^- + 2HCO_3^-$$

Methanol:

$$4CH_3OH + 2HCO_3^- \rightarrow 3CH_3COO^- + H^+ + 4H_2O$$

Aliphatic alcohols:

$$2R-CH_2OH + 2HCO_3^- \rightarrow 2R-COO^- + CH_3COO^- + H^+ + H_2O$$

1,2-Diols:

$$4R-CHOH-CH_2OH + 2HCO_3^- \rightarrow 4R-CH_2COO^- + CH_3COO^- + 3H^+ + 4H_2O$$

2,3-Butanediol:

$$4CH_3CHOH-CHOH-CH_3 + 6HCO_3^- \rightarrow 11CH_3COO^- + 5H^+ + 4H_2O$$

Acetoin:

$$2CH_3CO-CHOH-CH_3 + 2HCO_3^- \rightarrow 5CH_3COO^- + 5H^+ + 4H_2O$$

Glycerol:

$$CH_3OH-CHOH-CH_3OH + 2HCO_3^- \rightarrow 7CH_3COO^- + 5H^+ + 4H_2O$$

Lactate:

$$2CH_3CHOH-COO^- \rightarrow 3CH_3COO^- + H^+$$

Pyruvate:

$$4CH_3CO-COO^- + 4H_2O \rightarrow 5CH_3COO^- + 2HCO_3^- + H^+$$

Hexoses:

$$C_6H_{12}O_6 \rightarrow 3CH_3COO^- + 3H^+.$$

Substrate assimilated into cell material was calculated via acetate according to the following equation:

$$17CH_3COO^- + 11H_2O \rightarrow 8(C_4H_7O_3) + 2HCO_3^- + 15OH^-.$$

Growth yields were determined with all strains. Data for strain WoProp1 are listed in Table 2. With other strains basically the same results were obtained.

Effect of hydrogen on alcohol degradation

In coculture with the hydrogen-consuming *Methanospirillum hungatei*, strain WoProp1 fermented ethanol, propanol, butanol, and pentanol to the corresponding fatty acids as well as to acetate and hydrogen which was converted by *M. hungatei* to methane (Table 3). During fermentation of butanol and pentanol, more methane was formed than during ethanol and propanol degradation. The fermentation balance can be understood as a combination of the following two equations:

$$2R-CH_2OH + 2HCO_3^- \rightarrow 2R-COO^- + CH_3COO^- + H^+ + 2H_2O$$

$$2R-CH_2OH + HCO_3^- \rightarrow 2R-COO^- + CH_4 + H^+ + H_2O.$$

It appears that the ratio of methane over acetate formed was higher with long chain than with short chain alcohols.

Excess hydrogen added to a culture of strain WoProp1 growing on ethanol delayed and inhibited growth considerably (Fig. 4). Analysis of ethanol and hydrogen in the cultures indicated that ethanol rather than hydrogen was the substrate for growth in these cultures.

Discussion

Physiology

In the present study, new strains of strictly anaerobic bacteria are described which oxidized primary aliphatic alcohols to the corresponding fatty acids and used the resulting reducing equivalents for acetate synthesis from carbon dioxide. 1,2-Diols were oxidized in a similar manner. The strains also grew autotrophically on either formate or hydrogen/carbon dioxide. Sugars, some organic acids, acetoin, 2,3-butanediol and glycerol were converted to acet-

Table 1. Substrates tested for growth of alcohol fermenting strains of *Acetobacterium carbinolicum* compared with *A. woodii* and *A. wieringae*. Growth tests were carried out in defined medium, with *A. wieringae* in the presence of 0.1% yeast extract

Substrate degraded	*A. carbinolicum* strain		*A. woodii*[a]	*A. wieringae*[b]
	WoProp1	OttEtOH1		
H_2/CO_2	+	+	+	+
Formate	+	+	+	+
Methanol	+	−	+[c]	−
3,4,5-Trimethoxy-benzoic acid	+	+	+[c]	−[d]
3,4,5-Trimethoxy-cinnamic acid	+	+	+[c]	−[d]
Ethanol	+	+	−	−
Propanol	+	+	−	−[d]
Butanol	+	+	−	−[d]
Pentanol	+	+	−	−[d]
Ethyleneglycol	+	+	+	+[d]
1,2-Propanediol	+	+	+[d]	+[d]
2,3-Butanediol	+	−	+[e]	−[d]
Acetoin	+	+	+[e]	+[d]
Glycerol	+	+	+[d]	+
Malate	−	−	−	−
Lactate	+	+	±	+
Pyruvate	+	−	+	−
Glucose	+	−	−[f]	−
Fructose	+	−	+	+

± Means weak growth
[a] Data from Balch et al. 1977
[b] Data from Braun and Gottschalk 1982
[c] Data from Bache and Pfennig 1981
[d] Results of the present study
[e] Data from Schink 1984b
[f] Growth reported by Balch et al. (1977) could not be reproduced in our laboratory with strains DSM 1030 and NzVa16

ate as sole fermentation product. Methanol and methyl residues of methoxylated aromatic compounds were also fermented to acetate in a similar manner as observed with *Acetobacterium woodii* (Bache and Pfennig 1981), however, the double bond in cinnamic acid derivates was not reduced.

Among the homoacetogenic bacteria described so far, only *Clostridium aceticum* (Wieringa 1940; Adamse 1980; Braun et al. 1981) and *C. formicoaceticum* (Andreesen et al. 1970) are able to grow on ethanol, but higher alcohols are not utilized. Oxidation of 2,3-butanediol and acetoin was recently reported for *A. woodii*, *C. aceticum*, and *C. magnum* (Schink 1984b). Whereas most substrates were converted exclusively to acetate, oxidation of propanol, 1,2-propanediol, butanol, and pentanol by the new isolates led to formation of propionate, butyrate, and valerate, respectively, together with acetate which was formed as reduced fermentation product from carbon dioxide. Thus, homoacetogenesis should no longer be misinterpreted as a process in which acetate is the only end product; depending on the nature of the substrates, incomplete oxidation may lead to other products. Already the demethoxylation of methoxylated aromatic acids by *A. woodii* (Bache and Pfennig 1981) was, strictly speaking, such an incomplete oxidation. The basic property of all homoacetogenic bacteria is their ability to use carbon dioxide as inorganic electron acceptor, an ability which allows these bacteria to

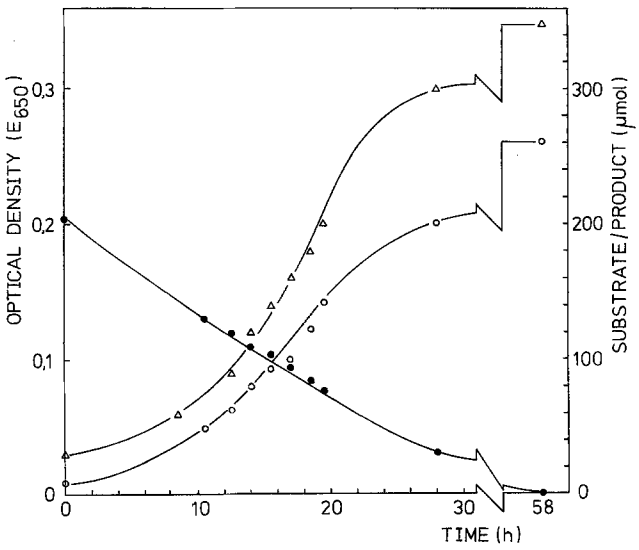

Fig. 3. Fermentation time course of *Acetobacterium carbinolicum* strain WoProp1 degrading ethanol. Experiments were performed at 28° C in 20 ml tubes sealed with butyl rubber septa. Samples were removed with a syringe at times indicated, and the headspaces were flushed with N_2/CO_2 gas mixture. Symbols △ cell density; ● ethanol degraded; ○ acetate formed. E_{650}, optical density at 650 nm

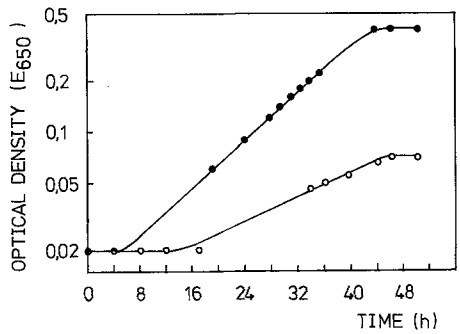

Fig. 4. Influence of H_2 on ethanol degradation by *Acetobacterium carbinolicum* strain WoProp1. Experimental conditions as described in Fig. 3. The medium contained 10 mmol/l ethanol and 0.05% yeast extract. Symbols ● growth under N_2/CO_2; ○ growth under H_2/CO_2. E_{650}, optical density at 650 nm

grow on a variety of reduced fermentation products and to use readily fermentable substrates (e.g. sugars) more efficiently than other fermenting anaerobes. We therefore basically agree with the principles Andreesen et al. (1970) formulated for homoacetogenic fermentations: that (1) three mol of acetate have to be formed from hexoses, (2) no (or only little) molecular hydrogen is formed, (3) carbon dioxide is necessary for growth and (4) carbon dioxide is incorporated into both carbon atoms of acetate, depending on the kind of substrate used. Coculture experiments with the new isolates and *Methanospirillum hungatei* demonstrated that part of the electrons derived from alcohol oxidation can be transferred to the methanogen, probably as molecular hydrogen. The amount of electrons transferred varied with the chain length of the alcohol: butanol and pentanol gave rise to more methane formation than ethanol and propanol. Since growth on butanol and pentanol is by far slower than

Table 2. Growth yields and stoichiometry of fermentation by *Acetobacterium carbinolicum* strain WoProp1

Substrate	Amount of substrate supplied (μmol)	Optical density (E_{650})	Cell dry weight formed[a] (mg)	Acetate assimilated[b] (μmol)	Products formed (μmol)				Growth yield (g per mol substrate utilized)	Carbon recovery[c] (%)
					Acetate	Propionate	Butyrate	Valerate		
H_2/CO_2	400	0.12	0.27	5.5	90.0	–	–	–	0.675	95.6
Formate	400	0.20	0.98	20.2	76.2	–	–	–	2.45	96.4
Methanol	100	0.17	0.82	16.9	62.4	–	–	–	8.20	105.7
3,4,5-Trimethoxy-benzoic acid	50	0.24	1.16	23.9	80.2	–	–	–	23.2	94.6
3,4,5-Trimethoxy-cinnamic acid	50	0.31	1.50	30.9	79.0	–	–	–	30.0	99.9
Ethanol	200	0.23	1.11	22.9	279.2	–	–	–	5.55	100.7
Propanol	200	0.21	1.02	20.9	81.3	195.2	–	–	5.10	99.1
Butanol	200	0.16	0.77	15.9	86.5	–	197.4	–	3.85	100.0
Pentanol	200	0.18	0.87	17.9	88.9	–	–	190.6	4.35	99.1
Ethyleneglycol	200	0.25	1.21	24.9	227.7	–	–	–	6.05	101.1
1,2-Propanediol	200	0.29	1.40	28.9	22.3	192.7	–	–	7.00	97.6
2,3-Butanediol	200	0.36	1.74	35.8	502.0	–	–	–	8.71	97.8
Acetoin	200	0.50	2.42	49.8	460.0	–	–	–	12.10	102.0
Glycerol	200	0.26	1.26	25.9	321.4	–	–	–	6.30	99.2
Lactate	200	0.30	1.43	29.5	276.6	–	–	–	7.14	102.0
Pyruvate	200	0.31	1.50	30.9	177.0	–	–	–	7.50	83.2
Glucose	50	0.50	2.42	49.9	106.0	–	–	–	48.4	103.9
Fructose	40	0.37	1.80	37.1	91.4	–	–	–	45.0	85.7

Experiments were carried out in 20 ml tubes which were completely filled. Growth on H_2/CO_2 was tested in half-filled tubes under an atmosphere of 80% H_2/20% CO_2

[a] Cell dry weights were calculated via cell density using the conversion factor 0.1 $OD_{650} \triangleq$ 24.2 mg dry weight per l, which was obtained by direct determination in 500 ml cultures grown with ethanol

[b] Substrate assimilated was calculated using the formula ($C_4H_7O_3$) for cell material

[c] Carbon recovery in pure cultures was calculated after the given equations of fermentation

Table 3. Growth yields and stoichiometry of fermentation by *Acetobacterium carbinolicum* strain WoProp1 in coculture with *Methanospirillum hungatei*

Substrate	Substrate supplied (μmol)	Acetate	Propionate	Butyrate	Valerate	Methane	Growth yield (g/mol)	Carbon recovery (%)
Ethanol	200	262	–	–	–	27	8.62	96.3
Propanol	200	70	189	–	–	27	8.62	95.3
Butanol	200	59	–	191	–	49	7.71	99.7
Pentanol	200	61	–	–	189	51	7.41	100.3

Experiments were carried out in half-filled 50 ml serum bottles, headspaces gassed with N_2/CO_2 gas mixture (80%/20%)
Calculation of yields and carbon recovery as described in Table 2

on ethanol or propanol, the methanogens appear to be more efficient in hydrogen scavenging in slow-growing systems than in fast ones. This observation is of importance with respect to natural environments: since subtrate turnover is much slower there than in laboratory cultures, homoacetogenic bacteria may act in nature as syntrophic partners of methanogens to a higher extent and with more substrates than expected so far (Winter and Wolfe 1979, 1980). Excess hydrogen added to pure cultures delayed and inhibited growth on ethanol considerably. Similar observations were made with *A. woodii* and *C. aceticum* growing on fructose (Braun and Gottschalk 1981) and can be explained as due to a shift in the redox state of cell-internal electron carriers. Thus, externally provided hydrogen inhibits ethanol oxidation not only in syntrophic mixed cultures but also in homoacetogenic pure cultures. Inhibition of ethanol degradation by hydrogen was recently demonstrated in sediments of Knaack Lake, an environment in which homoacetogenic bacteria similar to our isolates seem to be responsible for the bulk of ethanol degradation (Schink et al. 1985).

Taxonomy

The new bacteria described are typical homoacetogenic bacteria forming acetate as reduced end product either from organic substrates or from H_2/CO_2 or formate. Although they are physiologically similar to *Clostridium aceticum* they cannot be classified with this genus since they do not form spores. Morphological, cytological and most physiological properties are very similar to those of *Acetobacterium woodii*.

They have nearly identical G+C-contents of the DNA and contain murein of the same crosslinkage type B (Kandler and Schoberth 1979; Kandler, personal communication). The most significant difference of the new strains compared to *A. woodii* and *A. wieringae* (Braun and Gottschalk 1982) is their capacity to oxidize primary aliphatic alcohols to the respective fatty acids and acetate. For this reason, the described isolates are attributed to the genus *Acetobacterium* as a new species, *A. carbinolicum*.

A. carbinolicum sp. nov.

car. bi. no′ li. cum M. L. adj. referring to carbinols syn. alcohols. *carbinolicum* metabolizing alcohols.

Rod-shaped cells, 0.8–1.0 × 1.5–2.5 μm, with slightly pointed ends, single or in pairs. Motile. No spore formation. Gram-positive.

Chemoorganotroph or autotroph. Methanol, ethanol, 2,3-butanediol, acetoin, glycerol, lactate, pyruvate, glucose, fructose as well as methyl groups of methoxylated aromatic compounds and betaine utilized for growth and fermented to acetate. Propanol, butanol, pentanol and 1,2-propanediol fermented to the corresponding fatty acids and acetate.

Autotrophic growth on formate and H_2/CO_2; acetate formed. Sulfate, thiosulfate, elemental sulfur, or nitrate not reduced. No cytochromes.

Strictly anaerobic. Growth requires mineral medium with less than 0.7% (w/v) NaCl and 0.15% (w/v) $MgCl_2 \cdot 6H_2O$.

pH-range: 6.0–8.0, optimum at 7.0.

Temperature-range: 15° C–40° C, optimum at 27° C.

DNA base ratio 38.5 mol% ± 1.0% G+C.

Habitats: anoxic muds of freshwater origin.

Type strain: WoProp1, DSM 2925; deposited in Deutsche Sammlung von Mikroorganismen, Göttingen, FRG.

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