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New species of psychrophilic acetogens: *Acetobacterium bakii* sp. nov., *A. paludosum* sp. nov., *A. fimetarium* sp. nov.

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Abstract Three strains of new acetogenic bacteria were isolated from several low temperature environments. Cells were gram-positive, oval-shaped flagellated rods. The organisms fermented H_2/CO_2, CO, formate, lactate, and several sugars to acetate. Strains Z-4391 and Z-4092 grew in the temperature range from 1 to 30° C with an optimum at 20° C; strain Z-4290 grew in the range from 1 to 35° C with an optimum at 30° C. The DNA G+C content of strains Z-4391, Z-4092, and Z-4290 was 42.1, 41.7, and 45.8 mol%, respectively.

Key words Acetogenic fermentation · Anaerobic Psychrophilic · Psychrotrophic · *Acetobacterium bakii* *Acetobacterium paludosum* · *Acetobacterium fimetarium*

Introduction

Acetogenesis is a wide-spread microbial metabolic process occurring in various anoxic habitats, ranging from soils and sediments to gastrointestinal tracts of many animals (Drake 1992). At low temperatures, acetogenesis becomes one of the most appreciable microbial processes and acetogens might be considered as one of the most typical participants of the anaerobic decomposition community. Jones and Simon (1985) observed that at 10° C, hydrogen-utilizing acetogens from freshwater sediments consume an amount of hydrogen equivalent to 50% of that used by methanogens. Conrad et al. (1989) concluded that acetogenesis is the dominant process responsible for hydrogen turnover in anoxic paddy soil and sediment of mesotrophic Lake Constance at temperatures below 20° C. Acetogenic bacteria from several environments (anoxic sediments polluted with paper-mill waste water, tundra soil, cattle manure digested at low temperature) demonstrate a high ability to compete with methanogens for methanol and hydrogen-supplying substrates at temperatures of 15° C and below, resulting in accumulation of acetate during the first phase, followed by slow acetoclastic methanogenesis in the second phase (Kotsyurbenko et al. 1992; Parshina et al. 1993; Zavarzin et al. 1993; Nozhevnikova et al. 1994). These results show that the role of acetogenic bacteria in anaerobic processes in cold habitats may be of greater importance than assumed before.

So far, only one pure culture of psychrophilic acetogenic bacteria, strain HP4, has been isolated (Bak 1988). No other publications concerning the isolation of a pure culture of psychrotrophic or psychrophilic acetogenic bacteria exist. In this paper, we describe three new strains of psychrotrophic acetogenic bacteria isolated from several low temperature environments.

Materials and methods

Sources of organisms

The strains were enriched and isolated in pure culture from several environments: (1) Eight strains were isolated from the sediments of a pond polluted with paper-mill waste water (62 N, 51E; Kotsyurbenko et al. 1992; 1993b). The water temperature of the pond throughout most of the year was approximately 4–6° C. Of the eight strains, strain Z-4391 (DSM 8239) had the highest growth rate at 6° C and was selected for further investigation. (2) Strain Z-4092 (DSM 8237) was isolated from sediment of a fen located 100 km north of Moscow. When the sample was taken the temperature of the fen water was 1–2° C (Zhilina and Zavarzin 1991). (3) Five strains were isolated from cattle manure digested at 6° C for 1.5 years (Kotsyurbenko et al. 1993a). Of the five strains, strain Z-4290 (DSM 8238) had the highest growth rate at 6° C and was used for further investigation.

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Cultivation and pure culture isolation

The following medium was used for enrichment and further cultivation: mineral solution (Pfennig 1965), 0.05% $Na_2S \times 9H_2O$, 0.15% $NaHCO_3$, 0.002% resazurin, 0.02% yeast extract, 1 ml/l Lippert microelement solution (Pfennig and Lippert 1966), 10 ml/l vitamin solution (Wolin et al. 1963). Cultures were grown under a H_2/CO_2 (80:20) atmosphere at 6° C and at pH 6.5. For pure culture isolation, repeated transfers in the same medium were carried out using 1:10 serial dilutions. Growth of acetogenic bacteria was determined by acetate production and by cell counts using phase contrast microscopy. The final positive dilution was used to inoculate roll-tubes containing the same medium with 0.04% methanol and 2% agar. Colonies that developed in the roll-tube were transferred to tubes containing liquid medium.

Morphology

Cell morphology was studied by phase contrast and electron microscopy. The fine structure was studied using a JEM-100 electron microscope (Japan). Whole cells were shadowed with Na_2WO_4. To obtain thin sections, cells were fixed with 5% glutaraldehyde for 2 h at 4° C, then treated with 1% OsO_4 for 4 h at 4° C. Cells were embedded in epon-812, and thin-sections were stained by uranyl-acetate and lead citrate.

Physiology

Volatile products and gases were analyzed using a Chrom-5 gas chromatograph (former Czechloslavakia). H_2 and CO_2 were detected using a thermoconductivity detector and an activated charcoal AG-3 column. Acetate was analyzed using a flame ionization detector and a Chromosorb-101 (Serva, Heidelberg, Germany) column (oven temperature 170° C, injector temperature 200° C). The carrier gas was argon.

To estimate the pH-dependence of growth, the pH of the medium was adjusted by addition of a 10% solution of NaOH or H_2SO_4. To determine substrate utilization, the appropriate substrate was added at a concentration of 0.5% (w/v) to the mineral medium; gas phase was N_2/CO_2 (80:20). The organisms were cultivated under atmosphere of H_2/CO_2 (80:20) or CO (100%) when these compounds were used as the growth substrates. Sensitivity to antibiotics was determined by the addition of 100 µg antibiotic per ml of medium.

Lipid analysis

Cells (1.0 g) were dried under helium, then subjecteed to acid methanolysis in dry methanol plus 5.6N HCl for 4 h at 80° C. Fatty acids were extracted with hexane. The supernatant was dried and the residue was treated with BSTFA (bis-trimethylsisyl-trifluoracetamide). Derivates were analyzed with HP-5985B Hewlett-Packard chromatomass-spectrometer using a fused silica capillary column 25 m × 0.2 mm at 120–128° C (Janzen and Bryn 1985).

Determination of G + C content and DNA-DNA homology

DNA was isolated and purified from lysozyme-SDS-treated cells by the method of Marmur (1961). The G + C content of the DNA was determined from melting point analysis (Marmur and Doty 1962) by dissolving the purified DNA in 15 mM NaCl and 1.5 mM sodium citrate. DNA-DNA hybridization was carried out according to De Ley et al. (1970). For DNA-DNA hybridization analysis the following strains of *Acetobacterium* were used: *A. woodii* (DSM 1030), *A. wieringae* (DSM 1911), *A. carbinolicum* (DSM 2925), and *A. malicum* (DSM 4132).

Results

Cellular properties

The cells of all strains were oval-shaped short rods. The cellular morphology varied somewhat with the age of the cultures and the growth medium. Cells of actively growing cultures had an almost coccoid shape. Cells of older cultures were short rods. Cells growing on organic substrates were often irregular in form. Cells growing with H_2/CO_2 were arranged singly or in pairs; those growing with organic substrates occurred in chains of 3–20 cells. Cells of strain Z-4391 were 0.9–1.5 µm in width and 1.5–2.7 µm in length (Fig. 1). The size of cells of strain Z-4092 were 0.8–1.1 × 1.3–2.9 µm (Fig. 2) and of the strain Z-4290 were 0.8–1.1 × 1.5–2.6 µm (Fig. 3). The cells of all strains were motile, but lost motility in ageing cultures. Cells of strain Z-4391 had two subterminal flagella. Cells of strains Z-4092 and Z4290 had peritrichious flagella. The cell wall of all isolates had a gram-positive structure according to thin section electron microscopy (Figs. 1c, 2c, 3c). The cells of all strains divided by septum. Abundant slime production by all organisms was observed. None of our strains formed endospores. The cultures did not grow again after heating for 3 min at 100° C. The colonies of all strains were white, opaque, and 0.6–1.0 mm in diameter.

Table 1 Fatty acid and aldehyde composition of the lipid complex of strains Z-4391, Z-4092, and Z-4290 grown at 20° C

Chain length [a]		Strain		
Fatty acids	Aldehydes	Z-4391	Z-4290	Z-4092
$C_{12:0}$		–	1.5	–
$C_{14:1\Delta9}$		–	8.4	–
$C_{14:1\Delta11}$		–	7.0	–
$C_{14:0}$		2.4	8.0	–
	$C_{14:1\Delta9}$	–	2.3	–
	$C_{14:1\Delta11}$	–	1.6	–
	$C_{14:0}$	0.9	6.5	–
$C_{15:0}$		1.7	0.4	–
$C_{16:1\Delta9}$		12.4	12.3	14.0
$C_{16:1\Delta11}$		7.3	11.5	5.5
$C_{16:0}$		16.9	9.5	28.0
	$C_{16:1\Delta9}$	2.8	8.2	–
	$C_{16:1\Delta11}$	1.3	6.0	–
	$C_{16:0}$	34.3	13.1	17.7
$C_{17:0}$		–	–	0.5
	$C_{17:0}$	1.1	–	–
$C_{18:1\Delta9}$		5.0	1.2	8.3
$C_{18:1\Delta11}$		6.6	1.2	16.9
$C_{18:0}$		2.5	1.4	4.4
	$C_{18:1}$	2.1	–	–
	$C_{18:0}$	2.7	0.5	4.8

[a] First number represents length of carbon chain, second number refers to number of double bounds, third number represents the location of the double bound

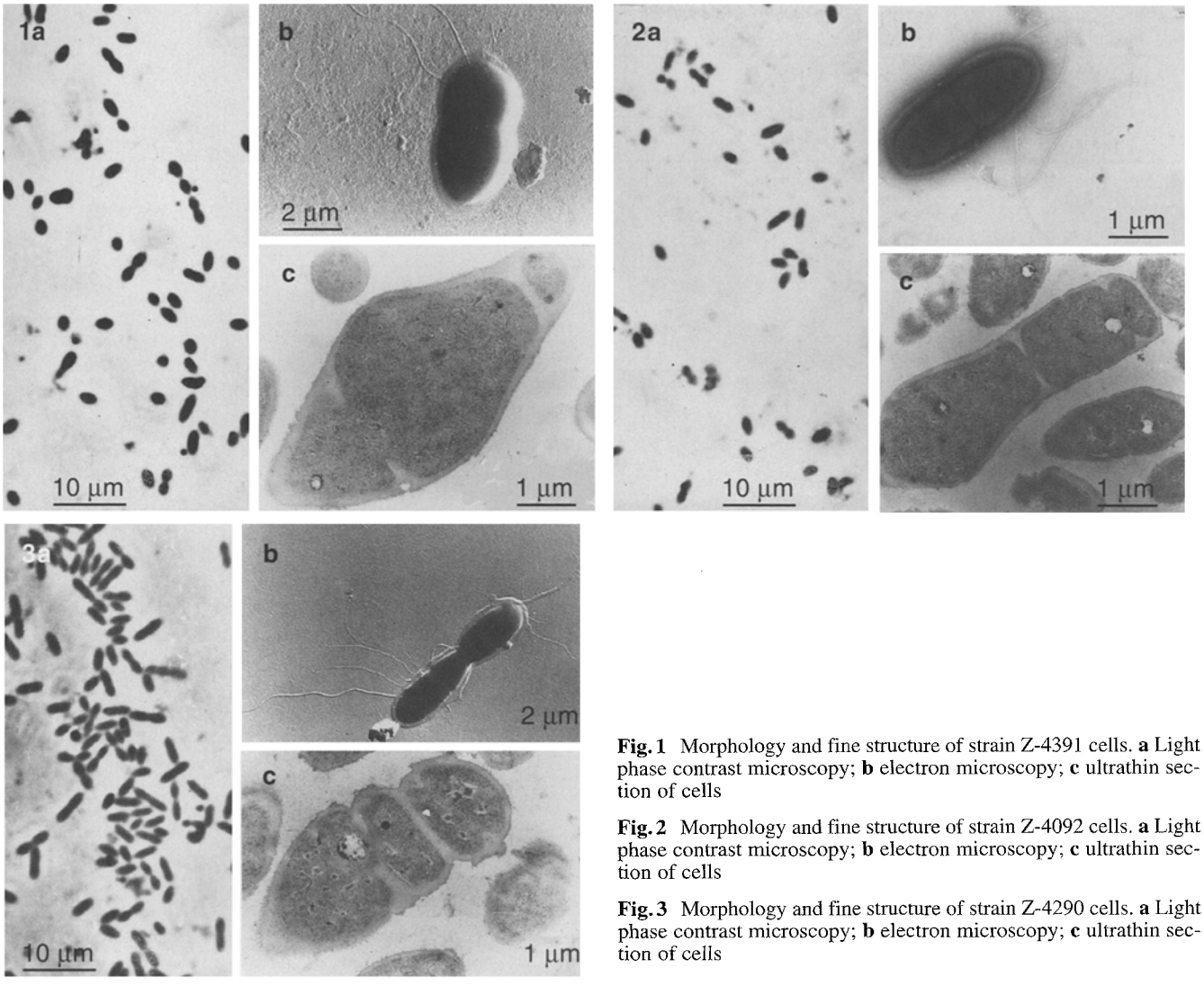

Fig. 1 Morphology and fine structure of strain Z-4391 cells. **a** Light phase contrast microscopy; **b** electron microscopy; **c** ultrathin section of cells

Fig. 2 Morphology and fine structure of strain Z-4092 cells. **a** Light phase contrast microscopy; **b** electron microscopy; **c** ultrathin section of cells

Fig. 3 Morphology and fine structure of strain Z-4290 cells. **a** Light phase contrast microscopy; **b** electron microscopy; **c** ultrathin section of cells

The main lipid complex component of strain Z-4391 was the saturated aldehyde of long-chain fatty acid $C_{16:0}$, obviously arising from alkyl-l-ethyl chains of plasmologens; that of strain Z-4092 was the saturated fatty acid $C_{16:0}$ (Table 1). The main components of the lipid complex of strain Z-4290 were unsaturated fatty acids $C_{16:1\Delta9}$ and the $C_{16:1\Delta11}$ and the saturated aldehyde $C_{16:0}$. The presence of unsaturated fatty acids $C_{14:1\Delta9}$ and $C_{14:1\Delta11}$ in the lipid complex of strain Z-4290 was the characteristic peculiarity of this organism. These components were absent in the lipid complex of strains Z-4391 and Z-4092.

Metabolic properties

Strains grew autotrophically on H_2/CO_2, formate, and CO. The organisms also utilized several organic substrates (Table 2). Strains Z-4193 and Z-4290 utilized some aromatic compounds. Acetate was the only end product when the isolates grew on H_2/CO_2, CO, formate, methanol, sugars or lactate.

Growth characteristics

All organisms were obligately anaerobic. Strains Z-4391 and Z-4092 grew from 1 to 30° C, with an optimum at 20° C. The optimal growth temperature of strain Z-4290 was 30° C, with growth occurring from 1 to 35° C (Fig. 4). All three strains had a high growth rate at low temperatures (1–10° C). The growth rate considerably decreased at temperatures higher than the optimum. Strain Z-4391 grew from pH 5.5 to 8.5, with an optimum at pH 6.5; strain Z-4092 grew from pH 5.0–8.0, with an optimum at pH 7; and strain Z-4290 grew from pH 6.0–8.5 with an optimum at pH 7.5.

The stoichiometry of hydrogen and carbon dioxide utilization with acetate formation was measured with all strains. Hydrogen and carbon dioxide were converted to acetic acid according to the following equation:

$$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$$

Growth of all strains was completely inhibited by streptomycin, benzylpenicillin, vancomycin, rifampicin, and bacitracin.

Table 2 Substrate specificity of new isolates and the species of the genus *Acetobacterium*. OD_{600} of freshly inoculated cultures was 0.005. (+*w* weak growth; ± growth of some strains; *ND* no data)

Substrate	Strain Z-4391	Strain Z-4290	Strain Z-4092	*A. woodii*[acde]	*A. wieringae*[bcd]	*A. carbinolicum*[cd]	*A. malicum*[d]
	Maximal OD_{600}						
H_2/CO_2	0.12	0.11	0.12	+	+	+	+
CO	0.10	0.10	0.10	+	ND	ND	ND
Formate	0.21	0.06	0.10	+	+	+	+w
Ramnose	–	–	–	ND	ND	ND	ND
Sucrose	–	–	–	ND	ND	ND	ND
Maltose	0.02	–	0.15	ND	ND	ND	ND
Melibiose	–	–	–	ND	ND	ND	ND
Lactose	–	–	–	ND	ND	ND	ND
Fructose	0.53	0.33	0.52	+	+	+	±
Glucose	0.03	–	0.21	–	–	+	–
Galactose	–	–	–	ND	ND	ND	ND
Xylose	0.04	–	0.05	ND	ND	ND	ND
Ribose	–	–	–	ND	ND	ND	ND
Arabinose	–	–	–	ND	ND	ND	ND
Raffinose	–	–	–	ND	ND	ND	ND
Ramnose	–	–	–	ND	ND	ND	ND
Cellobiose	–	–	0.05	ND	ND	ND	ND
Methanol	0.27	–	0.32	+	–	+	–
Ethanol	–	–	–	–	–	+	–
Propanol	–	–	–	–	–	+	–
Butanol	–	–	–	–	–	+	–
2,3-Butanediol	–	0.04	–	+	–	+	–
2-Methoxyethanol	0.03	0.04	0.04	–	–	–	+
Fumarate	–	–	–	–	–	–	–
Malate	0.18	0.25	0.10	–	–	–	+
Lactate	0.13	0.22	0.11	+w	+	+	+
Betaine	0.16	0.14	0.23	+	+	+	+
Gallate	0.09	–	–	ND	ND	ND	ND
Vanillate	0.08	0.08	–	ND	ND	ND	ND
Ethylene glycol	–	–	–	+	+	+	±
Trimethylamine	–	–	–	–	–	–	–

[a] Balch et al. (1977), Bache and Pfennig (1981)
[b] Braun and Gottschalk (1982)
[c] Eichler and Schink (1984)
[d] Tanaka and Pfennig (1988)
[e] Sharak Genther and Bryant (1987)

Genomic characteristics

The DNA G + C content was 42.1 mol% for strain Z-4391, 41.7 mol% for strain Z-4092, and 45.8 mol% for strain Z-4290. The results of DNA-DNA hybridization are presented in Table 3. DNA-DNA hybridization indicated a 21% homology between strains Z-4391 and Z-4092, 24% between strains Z-4391 and Z-4290, and 19% between strains Z 4092 and Z-4290.

Discussion

The new isolates were typical homoacetogens. Autotrophic growth occurred on H_2/CO_2, CO, and formate. The organisms converted certain sugars stoichiometrically to acetate during heterotrophic growth. Others volatile fatty acids or alcohols were not detected.

Strains Z-4391 and Z-4092 had an optimal growth temperature at 20° C and did not grow at temperatures above 30° C. According to the recognized definition of psychrophilic microorganisms given by Morita (1975), these bacteria should be classified as psychotrophs. Strain Z-4290 had an optimal growth temperature at 30° and should be classified as mesophile. On the other hand, strain Z-4290, as well as strains Z-4391 and Z-4092, were isolated at 6° C. Strain Z-4290 was well-adopted to low temperature conditions, was able to grow even at 1° C, and did not grow at temperatures above 35° C. According to the opinion of Russell (1990), Z-4290 could also be classified as psychrotrophic organism. The terminology of classification of anaerobic bacteria growing at low temperatures is not yet developed. Classification of Morita (1975) was proposed mainly for aerobic bacteria, in general having lower temperature growth optima than those of strict anaerobic bacteria. More information on anaero-

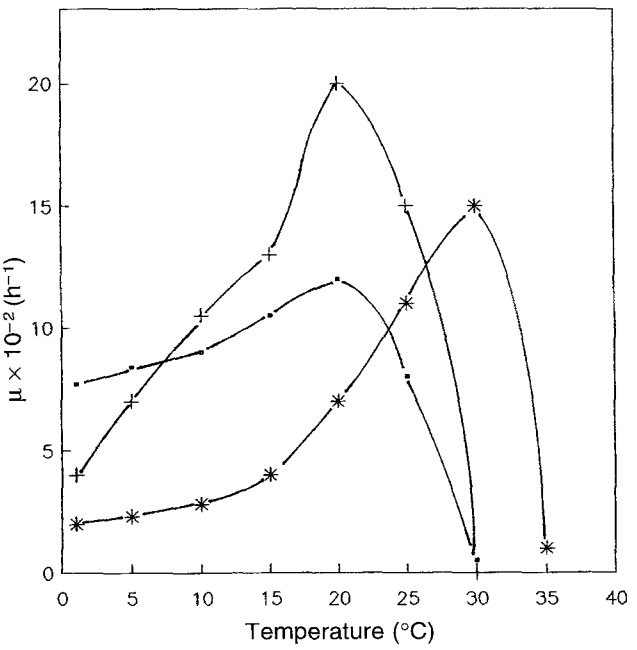

Fig. 4 Growth rate of the new isolates at various temperatures. *Dots* strain Z-4391; *Crosses* strain Z-4092; *Asterisks* strain Z-4290

bic microorganisms from low temperature habitats is needed. The name of "psychroactive" could also be proposed for mesophilic microorganisms able to grow at temperatures below 10°C.

The new isolates are different species. Cells of these bacteria differ in size and flagellation. The strains differ in their metabolic properties (Table 2), pH-limit and pH-optimum of growth, and DNA G + C content. Strains Z-4391 and Z-4092 differ also from the strain Z-4290 in their temperature optimum and limit of growth. The strains also differ considerably in the composition of the lipid complex. The results of DNA-DNA hybridization also confirm that the new isolates are different species.

Strains Z-4391, Z-4092, and Z-4290 are gram-positive, nonspore-forming bacteria with DNA G + C contents of 42.1, 41.7, and 45.8 mol% respectively, converting H_2 and CO_2, CO, formate, methanol, and sugars to acetate as the only end product. They could be classified with the genus *Acetobacterium* (Balch et al. 1977); however, these isolates differ from other described species of the genus *Acetobacterium* in their DNA G + C content, substrate specificity, and ability to grow at low temperature. A DNA homology between two strains of less than 60% indicates classification as different species (Grimont 1988). The results of DNA-DNA hybridization between the new isolates and the so far described species of the genus *Acetobacterium* (Table 3) clearly indicate that strains Z-4391, Z-4092, and Z-4290 are new species of this genus.

Lipid complex composition has been proposed as an important characteristic of bacterial species (Russell 1990; Zhilina et al. 1992). Unfortunately, no data are available on lipid complex composition of other species of the genus *Acetobacterium*. The prevalence of $C_{16:0}$ fatty acids and aldehydes in the lipid complex of the new isolates seems to be a general feature of these bacteria.

So far, only one psychrotrophic acetogenic bacterium, *Acetobacterium* strain HP4, has been isolated (Conrad et al. 1989). This organism was assigned as *A. carbinolicum*, although its growth temperature considerably differs from that of the type strain (Eichler and Schink 1984), and it is not known if strain HP4 utilizes propanol, butanol, and other aliphatic alcohols as does *A. carbinolicum*. It is difficult to compare our isolates with strain HP4 as a complete description of this organism is lacking. However, cells of strain HP4 are nonmotile, while our isolates are motile. Unlike strain HP4, our bacteria do not utilize ethylene glycol. For these reasons, we designate the isolates Z-4391, Z-4092, and Z-4290 as new species of the genus *Acetobacterium* and name them *A. bakii*, *A. paludosum*, and *A. fimetarium*, respectively.

Description of *Acetobacterium bakii* sp. nov.

ba'kii – L. n., *Bakii* – named after Friedhelm Bak, who isolated the first psychrotrophic acetogenic bacterium.

Short nonspore-forming rods, 0.9–1.5 × 1.5–2.7 μm. Motile by means of two subterminal flagella. Gram-positive. Main lipid complex component of cells is a saturated aldehyde $C_{16:0}$. Chemoorganotroph or autotroph. Maltose, fructose, glucose, xylose, lactate, malate, 2-methoxyethanol, methanol, and methyl groups of aromatic compounds and betaine utilized for growth and fermented to acetate. Autotrophic growth on H_2/CO_2, formate, and CO; acetate formed. Psychrotrophic, optimal growth temperature 20°C, maximum 30°C, minimum 1°C. pH growth

Table 3 DNA-DNA hybridization of *Acetobacterium* strains

Organism	DNA-DNA hybridization (%)						
	A. woodii	*A. carbinolicum*	*A. wieringae*	*A. malicum*	Z-4391	Z-4092	Z-4290
A. woodii	100						
A. carbinolicum	69	100					
A. wieringae	28	36	100				
A. malicum	15	26	30	100			
Z-4391	27	22	29	20	100		
Z-4092	13	18	16	16	21	100	
Z-4290	24	23	25	14	24	19	100

range pH 5.5–8.5, optimum pH 6.5. Strictly anaerobic. DNA base ratio 42.1 ± 1 mol%. Isolated from anaerobic sediment of a pond polluted by paper-mill waste water. Type strain: Z-4391.

Description of *Acetobacterium paludosum* sp. nov.

Pa. lu. do'sum – L. adj. *n.*, fen, *paludosum* – isolated from fen.

Short nonspore-forming rods, 0.8–1.1 × 1.3–2.9 μm. Motile by means of peritrichous flagella. Gram-positive. Main lipid complex component of cells is a saturated fatty acid $C_{16:0}$. Chemoorganotroph or autotroph. Maltose, fructose, glucose, xylose, cellobiose, lactate, malate, 2-methoxyethanol, methanol, and methyl groups of betaine utilized for growth and fermented to acetate. Autotrophic growth on H_2/CO_2, formate, and CO; acetate formed. Psychrotrophic, optimal growth temperature 20° C, maximum 30° C, mimimum 1° C. pH growth range pH 5.0–8.0, optimum pH 7.0. Strictly anaerobic. DNA base ratio 41.7 ± 1 mol%. Isolated from anaerobic sediment of a fen. Type strain: Z-4092

Description of *Acetobacterium fimetarium* sp. nov.

fi.me.ta'ri.um – L. adj. n., *fimetarius* – inhabiting manure, *fimetrium* isolated from manure.

Short nonspore-forming rods, 0.8–1.1 × 1.5–2.6 μm. Motile by means of peritrichous flagella. Gram-positive. Characteristic peculiarity of lipid complex of cells is the presence unsaturated fatty acids $C_{14:1\Delta9}$ and $C_{14:1\Delta11}$. Main lipid complex components of cells are unsaturated fatty acids $C_{14:1\Delta9}$ and $C_{16:1\Delta11}$ and a saturated aldehyde $C_{16:0}$. Chemoorganotroph or autotroph. Fructose, lactate, malate, 2-methoxyethanol, 2,3-buthanediol, and methyl groups of vanilate and betaine utilized for growth and fermented to acetate. Autotrophic growth on H_2/CO_2, formate, and CO; acetate formed. Psychrotrophe or psychroactive mesophile, optimal growth temperature 30° C, maximum 35° C, minimum 1° C. pH growth range pH 6.0–8.5, optimum pH 7.5. Strictly anaerobic. DNA base ratio 45.8 ± 1 mol%. Isolated from digested manure. Type strain: Z-4290.

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