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ACETOBACTER POLYOXOGENES SP. NOV., A NEW SPECIES OF AN ACETIC ACID BACTERIUM USEFUL FOR PRODUCING VINEGAR WITH HIGH ACIDITY

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Fourteen strains of acetic acid bacteria were isolated from fermented vinegar broth with high acidity. These isolates were useful for producing high-acidity vinegar and require acetic acid, ethanol and glucose for growth. The isolates differed from all the species of the genera *Acetobacter* and *Gluconobacter* so far validly published, with respect to their morphological, physiological, biochemical and chemotaxonomic characters' and their deoxyribonucleic acid homologies. The isolates are classified as a new species of the genus *Acetobacter*, and we propose *Acetobacter polyoxogenes* sp. nov. for them. The type strain of *A. polyoxogenes* is NBI 1060 (=JCM 3808).

There are several methods in Japan for the industrial production of vinegar by acetic acid bacteria. They are generally divided into two methods, a traditional method by surface culture and a modern method by submerged culture. The final acidity of the fermented vinegar is usually less than 10%.

Purely isolated strains of the acetic acid bacteria have seldom been used for industrial production of vinegar in Japan. So called "seed vinegar," a mixture of heterogeneous microorganisms, is usually employed as a starter. Different kinds of spontaneous development of acetic acid bacteria occur in each of the methods mentioned above. The dominant acetic acid bacteria in such vinegar production were reported to be *Acetobacter pasteurianus* in the surface culture and *Acetobacter xylinum* in the submerged culture (*1*). However, the acidity produced by these bacteria is not very high.

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There is another method which uses submerged culture to produce vinegar with high acidity of more than 10%. This method is industrially important. However, the microorganisms have not been isolated from fermented vinegar broth with high acidity, because the bacteria could not grow on the ordinary media used for the usual acetic acid bacteria.

The present study deals with the isolation and characterization of acetic acid bacteria which produce high-acidity vinegar. The isolates are classified as a new species of the genus *Acetobacter*. A new species, *Acetobacter polyoxogenes*, is proposed for these strains.

MATERIALS AND METHODS

Isolation of bacteria. The medium developed and employed for the isolation was an acetic acid-ethanol (AE) agar plate which was AE broth solidified by adding 0.5% agar. Its surface was further coated with AE broth with 1% agar. The AE broth was composed of 1.5% glucose, 0.2% yeast extract, 0.3% peptone, 6.5% acetic acid and 2% (v/v) ethanol. The acetic acid and ethanol were added asepctically. The final pH of the AE broth was not adjusted but was approximately 2.9. Samples for isolation were various kinds of fermented vinegar broth collected from some vinegar breweries. The samples were spread on AE agar plates and incubated at 30°C for 7 to 30 days in a chamber kept at 95 to 100% relative humidity. Microorganisms appearing on AE agar plates were picked up and purified by repeated streak cultures on AE agar plate. When plates with the usual agar strength (1.5 to 2.0%) were used for isolation from fermented vinegar broth with high acidity, no microorganisms were obtained. Cultivation on the double-layer agar plate under high humidity is necessary for the formation of the high-acidity bacterial colonies. The soft-agar lower layer apparently supplies a wet environment to the surface layer.

Bacterial strains. In addition to the isolates mentioned above, the following strains were used for comparison: *Acetobacter liquefaciens* IAM 1834^T, *Acetobacter xylinum* NCIB 11664^T, IFO 3288, NBI 1002 and NBI 1003, *Acetobacter hansenii* NCIB 8746^T, *Gluconobacter cerinus* IFO 3267^T, *Gluconobacter oxydans* subsp. *oxydans* ATCC 19357^T, *Gluconobacter oxydans* subsp. *suboxydans* IFO 3990, *Gluconobacter oxydans* subsp. *industrius* IFO 3260, *Gluconobacter oxydans* subsp. *melanogenes* IFO 3293, and *Gluconobacter oxydans* subsp. *sphaericus* IFO 12467^T. The superscript "T" indicates strains that are the type strains of the species and subspecies.

Cultivation media. The isolates were cultivated on AE agar or AE broth at 30°C. In the cultivation on AE agar plates, the relative humidity was kept at 95 to 100%. Unless otherwise stated, the authentic strains were cultivated on glucose-yeast extract-peptone (GYP) agar containing 3% glucose, 0.5% yeast extract, 0.2% peptone and 1.5% agar.

Morphological characteristics. Cell shape was observed with an optical microscope using cells stained with carbol fuchsin. Cell shape was also observed with a transmission electron microscope JEOL model JEM-1200EX. Gram staining was carried out by HUCKER's modification (*2*). Cells grown on AE agar plates for 5 to 10 days were used for the observations. Motility and flagellation were observed in the cells cultured on AE agar plate for 4 to 30 days at 15, 20, 25 and 30°C. The presence of flagella was determined by the staining method of TODA (*3*).

Cultural characteristics. The colonial appearance of the isolates on AE agar plate was observed after incubation for 10 days. Growth on nutrient agar plates, GYP agar plates, YM agar plates and AE agar plates was observed after incubation for 30 days. YM agar was composed of 1% glucose, 0.5% peptone, 0.3% malt extract and 1.5% agar. Growth in liquid broth of these media was also studied.

Physiological and biochemical characteristics. The effect of pH on growth of the isolates was examined by their growth after incubation for 21 days on AE agar plates adjusted to pH 1.6, 1.9, 2.2, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0. The pH was adjusted with HCl and NaOH. Effect of temperature on growth of the isolates was tested by their growth on AE agar plates at 10, 15, 20, 25, 30, 35 and 40°C. The effect of acetic acid concentration on the growth of the isolates was determined by growing them on AE agar plates with different concentrations of acetic acid. The isolates were incubated for 21 days on media containing 0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, and 14% of acetic acid. The pH of these media was 6.15, 3.55, 3.36, 3.22, 3.03, 2.92, 2.84, 2.77, 2.71, and 2.66, respectively. The authentic strains were incubated for 21 days at 30°C on GYP agar slants containing 0, 0.5, 1.0, 2.0, and 4.0% acetic acid. Growth of the isolates on medium without acetic acid, ethanol or glucose was observed after growing for 21 days on AE agar plates lacking these compounds.

The utilization of various carbon compounds was examined by the growth on media based on AE agar. Since the isolates did not grow without acetic acid, ethanol and glucose, the test was as follows: The glucose in the AE agar was replaced consecutively by either L-arabinose, D-xylose, D-mannose, D-fructose, D-galactose, maltose, sucrose, lactose, raffinose, melibiose, trehalose, L-sorbose, D-sorbitol, D-mannitol, inositol, glycerol, or starch. The ethanol in the AE agar was replaced consecutively by either methanol, *n*-propanol, *iso*-propanol, *n*-butanol, *iso*-butanol, *n*-amyl alcohol, or *iso*-amyl alcohol. The carbon sources were sterilized separately by filtration, and added to the sterile media to a final concentration of 2%. The cultures were incubated for 21 days.

Production of acetic acid from ethanol and oxidation of ethanol to CO_2 and H_2O were tested as follows: The isolates were cultivated in AE broth with agitation and aeration for 21 days. The concentrations of acetic acid and ethanol during the cultivation were determined with a Seishin model S603 carboxylic

acid analyzer (Seishin Pharmaceutical Co., Ltd., Noda, Japan) and a Shimadzu GC-6A gas chromatograph (Shimadzu Co., Kyoto, Japan), respectively. Oxidation of lactate to CO_2 and H_2O was determined by the method of SHIMWELL et al. (*4*). LEIFSON's method (*5*) was used to test the reaction in acetate and lactate. Oxygen consumption with acetate, lactate and succinate were detected by the method of AMEYAMA and KONDO (*6*).

For the test of formation of gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid, and 2, 5-diketogluconic acid, the isolates were cultivated in AE broth supplemented with 1.5% of glucose with shaking for 4, 7, 14 and 21 days. Gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid and 2,5-diketogluconic acid were detected by thin-layer chromatography (*7*) and quantitatively determined with the carboxylic acid analyzer mentioned above.

Dihydroxyacetone from glycerol was tested as follows: After the isolates were streaked on AE agar plates with and without glucose which were supplemented with 3% glycerol then incubated for 4, 7, 14 and 21 days, an aliquot of Fehling's solution was poured onto the two kinds of plates. An orange color occurred around the streak when the strain oxidized glycerol to dihydroxyacetone.

For the test of ferric chloride reaction in glucose and fructose, AE broth supplemented with 3.5% of glucose and AE broth containing 5% fructose in place of glucose were used. After incubation for 4, 7, 14 and 21 days with shaking, the broth was tested for the reddish violet color reaction with aqueous ferric chloride solution.

For the test of cellulose formation, the isolates were cultivated on AE agar plates for 21 days. The resulting colonies were stained with Lugol's iodine and applied with 60% H_2SO_4. Colonies were stained bright blue in the strains that formed cellulose.

AE agar plates were used to observe the formation of brown pigment by the isolates after incubation for 21 days. Growth on Frateur's modified Hoyer medium-ethanol and Frateur's modified Hoyer medium-glucose was observed by the method of SWINGS et al. (*8*). Both of the media were supplemented with the following vitamins. One liter of the media contained 2 μg of biotin, 400 μg of calcium pantothenate, 2 μg of folic acid, 2 mg of inostiol, 400 μg of niacin, 200 μg of *p*-aminobenzoic acid, 400 μg of pyridoxin HCl, 200 μg of riboflavin, and 400 μg of thiamine HCl. The biochemical tests of catalase, oxidase, formation of H_2S, and gelatin liquefaction of the isolates were examined by the method described by KOMAGATA (*9*), except that AE agar plate and AE broth were used as cultivation media. The following biochemical tests for the authentic strains were carried out by the method described in the previous report (*10*): Oxidation of ethanol via acetic acid to CO_2 and H_2O, formation of 5-ketogluconic acid and 2, 5-diketogluconic acid, dihydroxyacetone from glycerol, ferric chloride reaction in glucose and fructose, formation of cellulose, and formation of brown pigment.

Isoprenoid quinones. The isolates were cultivated in AE broth for 2 to 4 days at 30°C with shaking. Ubiquinones were extracted and purified according to the method of YAMADA et al. (*11*). The ubiquinone system was determined by the method described in the previous report (*10*).

Cellular fatty acid composition. Cells cultured in AE broth for 2 to 4 days with shaking were used for the analysis of cellular fatty acids. The cellular fatty acid composition was determined by the method of IKEMOTO et al. (*12*).

DNA base composition. DNA was purified by the method of SAITO and MIURA (*13*) from cells grown in AE broth with shaking for 2 to 4 days. The DNA base composition (G+C content) was calculated from the melting temperature (*14*) with a Beckman model DU-8 spectrophotometer (Beckman Instruments Inc., Fullerton, Calif., U.S.A.). DNA from *Escherichia coli* K12 (51.0 mol% of guanine plus cytosine (mol% G+C)) was used as a reference (*15*).

DNA-DNA homologies. DNA-DNA hydridization experiments were carried out at 70°C by the method previously reported (*16*). DNAs were labeled using the Nick Translation System (NEK. 005, New England Nuclear, Boston, Mass., U.S.A.).

Production of acetic acid by the isolates. The final concentration of acetic acid produced by the purified isolates was examined using fed-batch cultures as follows: Two liters of broth containing 1.5% glucose, 0.2% yeast extract, 0.3% peptone, 6.5% acetic acid, and 5.5% (v/v) ethanol were charged in a 3-liter fermentation apparatus. The broth was agitated with aeration at a rate of 0.5 vm (volume of air volume of medium^{-1} min^{-1}) at 30°C. Cells grown on AE agar plates for 4 to 7 days were used for inocula. After an acetic acid concentration increased to 8.5%, feed broth containing 1.5% glucose, 0.2% yeast extract, 0.3% peptone, and 50% (v/v) ethanol was supplied to the cultivating broth with a feed pump. Cultivation temperature was kept at 30°C until the acetic acid concentration reached 12.5%. Thereafter, the temperature of the broth was gradually lowered to 21°C according to the increase of acidity.

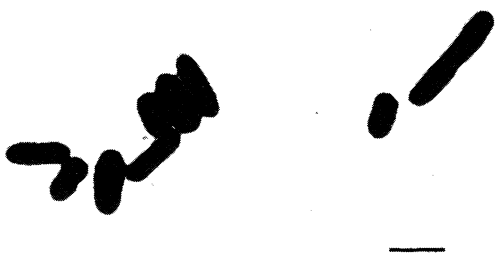

Fig. 1. Electron micrograph of the isolate NBI 1029. Scale indicates 1 μm.

Table 1. Comparison of characteristics of the fourteen

Characteristic	Isolates	*A. liquefaciens* IAM 1834^T	*A. xylinum* NCIB 11664^T	*A. xylinum* IFO 3288
Oxidation of ethanol to CO_2 and H_2O	−[c] (0/14)[d]	+[a]	+	+
Oxidation of lactate to CO_2 and H_2O	− (0/14)	+	+	+
Reaction in: acetate	− (0/14)	+	+	+
lactate	− (0/14)	+	+	+
Oxygen consumption for: acetate	− (0/14)	+	+	+
succinate	− (0/14)	+	+	+
Formation of: 5-ketogluconic acid	− (0/14)	+	w[b]	+
2,5-diketogluconic acid	− (0/14)	+	−	−
Dihydroxyacetone from glycerol	− (0/14)	+	+	+
Ferric chloride reaction in: glucose	− (0/14)	+	−	−
fructose	− (0/14)	+	−	w
Formation of cellulose	− (0/14)	−	+	+
Formation of brown pigment	− (0/14)	+	−	−
Growth on Frateur's modified Hoyer medium-ethanol (vitamin added)	− (0/14)	+	−	−
Range of acetic acid concn. for growth (%)	4–10(7/14) 4–12(1/14) 6–10(2/14) 6–12(4/14)	0–0.5	0–1	0–1
Growth at pH 4	− (0/14)	+	+	+
Growth on: medium not containing acetic acid	− (0/14)	+	+	+
medium not containing ethanol	− (0/14)	+	+	+

[a] positive.
[b] weakly positive.

RESULTS

Isolation of bacteria

Fourteen strains of bacteria, i.e., strains NBI 1028, NBI 1029, NBI 1036, NBI 1037, NBI 1038, NBI 1039, NBI 1041, NBI 1042, NBI 1043, NBI 1044, NBI 1045, NBI 1057, NBI 1058, NBI 1060, were isolated from fermented vinegar broth with high acidity.

Morphological characteristics

All of the isolates were gram-negative, non-motile, non-flagellated and non-sporeforming rods. The cell size of all the isolates was approximately 0.4 to 0.6 μm wide by 0.8 to 1.3 μm long. An electron micrograph of strain NBI 1029 is shown in Fig. 1.

Cultural characteristics

The surface colonies of the isolates on AE agar were punctiform, glossy,

isolates with those of acetic acid bacteria with Q-10.

A. hansenii NCIB 8746[T]	*G. cerinus* IFO 3267[T]	*G. oxydans* subsp. *oxydans* ATCC 19357[T]	*G. oxydans* subsp. *suboxydans* IFO 3990	*G. oxydans* subsp. *industrius* IFO 3260	*G. oxydans* subsp. *melanogenes* IFO 3293	*G. oxydans* subsp. *sphaericus* IFO 12467[T]
+	−	−	−	−	−	−
+	−	−	−	−	−	−
+	−	−	−	−	−	−
+	−	−	−	−	−	−
+	−	−	−	−	−	−
+	−	−	−	−	−	−
w	+	+	+	+	+	+
−	−	−	−	−	+	+
+	+	+	+	+	+	+
−	−	−	−	−	+	+
−	+	+	+	+	+	+
−	−	−	−	−	−	−
−	−	−	−	−	+	+
−	−	−	−	−	−	−
0–2	0–0.5	0–0.5	0–05	0–0.5	0–0.5	0–0.5
+	+	+	+	+	+	+
+	+	+	+	+	+	+
+	+	+	+	+	+	+

[c] negative.
[d] number of strains positive/number of strains tested.

smooth and light grey to light brown in color.

All the isolates grew on AE agar plate only in an atomosphere with a relative humidity of 95 to 100% and in AE broth only by shaking or agitation with aeration. They did not grow on or in any of the nutrient agar plates, nutrient broth, GYP agar plates, GYP broth, YM agar plates and YM broth under any of the given culture conditions.

Physiological and biochemical characteristics

The isolates grew at pH 2.2 to 3.5 and at 15 to 35°C. Acetic acid concentration for growth of the isolates ranged from 4 to 12%. Table 1 shows the comparison of the characteristics of the isolates with those of authentic strains.

Utilization of various carbon sources of the isolates is shown in Table 2. In the tests, all of the isolates grew on D-glucose, D-fructose, sucrose, D-sorbitol, D-mannitol, ethanol, and *n*-propanol. Almost all of them grew on *iso*-propanol. All of them produced acetic acid from ethanol, gluconic acid (>0.3%) and 2-

Table 2. Utilization of carbon compounds by the fourteen isolates.

Strain	Utilization of:																									
	L-Arabinose	D-Xylose	D-Glucose	D-Mannose	D-Fructose	D-Galactose	Maltose	Sucrose	Lactose	Raffinose	Melibiose	Treharose	L-Sorbose	D-Sorbitol	D-Mannitol	Inositol	Glycerol	Starch	Methanol	Ethanol	*n*-Propanol	*iso*-Propanol	*n*-Butanol	*iso*-Butanol	*n*-Amyl alcohol	*iso*-Amyl alcohol
NBI 1028	−[c]	−	+[a]	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−	−	−
NBI 1029	−	−	+	−	+	−	−	+	−	−	−	−	−	+	w[b]	−	−	−	−	+	+	+	−	−	−	−
NBI 1036	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	w	−	−
NBI 1037	−	−	+	−	+	−	−	+	−	−	−	−	−	+	w	−	−	−	−	+	+	+	−	−	−	−
NBI 1038	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	w	−	−
NBI 1039	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	w	−	−	−	−
NBI 1041	−	−	+	−	w	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	w	−	−	−	−
NBI 1042	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	w	−	−	−	−
NBI 1043	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	−	−	−	−	−
NBI 1044	−	−	+	−	+	−	−	+	−	−	−	−	−	+	w	−	−	−	−	+	+	−	−	−	−	−
NBI 1045	−	−	+	−	+	−	−	+	−	−	−	−	w	+	+	−	−	−	−	+	+	−	−	−	−	−
NBI 1057	−	−	+	−	w	−	−	+	−	−	−	−	w	+	+	−	−	−	−	+	+	w	−	−	−	−
NBI 1058	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−	−	−
NBI 1060	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−	−	−

[a] positive.
[b] weakly positive.
[c] negative.

These isolates required acetic acid, ethanol and glucose for growth. Therefore, utilization of carbon compounds was examined by the replacement of glucose and ethanol in the AE agar by other carbohydrates and alcohols. The carbon compounds listed in the table does not imply that these are the sole carbon sources in this test. See text for details.

ketogluconic acid (<0.1%), and they produced catalase. All of the isolates negatively reacted in the following tests: Oxidation of ethanol and lactate to CO_2 and H_2O; reaction in acetate and lactate; oxygen consumption for acetate, lactate and succinate; formation of 5-ketogluconic acid and 2, 5-diketogluconic acid; dihydroxyacetone from glycerol; ferric chloride reaction in glucose and fructose; formation of cellulose; formation of brown pigment; growth on Frateur's modified Hoyer medium-ethanol (vitamin added) and Frateur's modified Hoyer medium-glucose (vitamin added); growth on AE medium without any acetic acid, ethanol or glucose; oxidase; nitrate reduction; formation of H_2S; and gelatin liquefaction.

Isoprenoid quinones

A ubiquinone homologue with ten isoprene units (Q-10) was the major component of isoprenoid quinone in the cells of isolates NBI 1028, NBI 1036, NBI 1037, and NBI 1060 (Table 3).

Cellular fatty acid composition

The cellular fatty acid composition of four strains of the isolates are shown in Table 3. They contained a large amount of octadecenoic acid with straight-chain ($C_{18:0}$). There was also a small amount of straight-chain saturated $C_{16:0}$ acid, 2-OH-$C_{14:0}$ acid, and straight-chain saturated $C_{14:0}$ acid.

DNA base composition

The base compositions of the DNA of the four isolates, NBI 1028, NBI 1036, NBI 1037, and NBI 1060 were 57.9, 58.0, 57.6, and 58.1 mol% G+C, respectively.

DNA-DNA homologies

Table 4 shows the results of DNA-DNA hybridization. With the DNA of the isolate NBI 1028 as a reference, high homology indexes (100 to 92%) were obtained among the four isolates. However, the other five strains had low homology indexes (48 to 11%) to the isolate NBI 1028. In the reversed experiments using the labeled DNA of *A. liquefaciens* IAM 1834^T, *A. xylinum* IFO 3288 and *G. oxydans* ATCC 19357^T, the homology indexes were also low for each of the four isolates (16 to 13%, 39 to 34% and 19 to 15%, respectively).

Table 3. Isoprenoid quinone and cellular fatty acid composition of some isolates.

Strain	Isoprenoid quinone	Fatty acid composition (%)								
		14:0	16:0	16:1	17:0	18:0	18:1	2OH-14	3OH-14	2OH-16
NBI 1028	Q-10	1.3	13.9	1.0	tr[a]	5.4	65.5	4.8	tr	7.3
NBI 1036	Q-10	1.3	10.2	tr	—[b]	4.7	69.5	3.9	—	10.1
NBI 1037	Q-10	1.8	10.5	tr	—	5.3	66.7	3.3	—	11.9
NBI 1060	Q-10	1.8	8.9	tr	tr	5.0	69.5	3.6	tr	10.3

[a] trace (1.0%).
[b] not detected.

Table 4. DNA homologies of the isolates and some acetic acid bacteria with Q-10.

Strain	DNA homology (%) with			
	Isolate NBI 1028	*A. liquefaciens* IAM 1834^T	*A. xylinum* IFO 3288	*G. oxydans* ATCC 19357^T
Isolate NBI 1028	100	13	35	15
Isolate NBI 1036	95	15	34	16
Isolate NBI 1037	92	13	34	19
Isolate NBI 1060	95	16	39	18
A. liquefaciens IAM 1834^T	11	100	20	18
A. xylinum IFO 3288	39	16	100	19
A. xylinum NBI 1002	48	17	65	20
A. xylinum NBI 1003	38	19	63	20
G. oxydans ATCC 19357^T	17	18	24	100

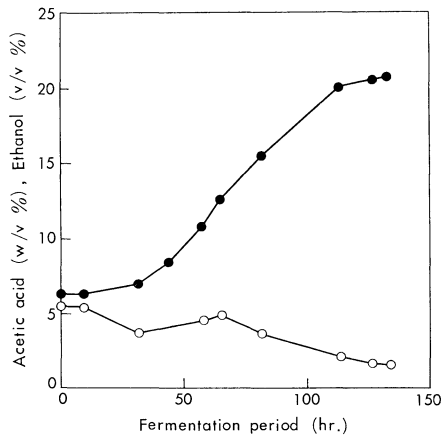

Fig. 2. Time course of acetic acid fermentation by the isolate NBI 1028.
Symbols used in the graph are: ●, acetic acid concentration (w/v %); ○, ethanol concentration (v/v %).

Acetic acid production by the isolates

All the isolates produced more than 15% acetic acid in the fed-batch cultivation. The pure culture of the isolates produced high concentration of acetic acid. For example, the isolate NBI 1028 produced 21% of acetic acid (Fig. 2).

DISCUSSION

All of the isolates were gram-negative, obligately aerobic and acidophilic rods and they oxidized ethanol to acetic acid. According to the 8th ed. of Bergey's Manual of Determinative Bacteriology (*17*), Bergey's Manual of Systematic Bacteriology Vol. 1 (*18–20*) and recent taxonomic studies (*8*, *21–23*), these strains

should be classified as a species of one of the genera *Acetobacter*, *Gluconobactar* and *Frateuria*.

The isolates had very high tolerance to acetic acid and ability to produce acetic acid from ethanol (Fig. 2), but their tests were negative for oxidation of ethanol and lactate to CO_2 and H_2O, reaction in acetate and lactate, and oxygen consumption for acetate, lactate and succinate (Table 1). Thus, the isolates had phenotypic characteristics intermediate between the genera *Acetobacter* and *Gluconobacter*. Furthermore, they had distinctive cultural features such as requiring media with at least 4% of acetic acid concentration (Table 1) and pH 2.2 to 3.5 for growth.

All the isolates contained Q-10 as a major isoprenoid quinone. Q-10 was found in the cells of *A. xylinum*, the peritrichously flagellated intermediate strains (*A. liquefaciens*), and all of the strains of *Gluconobacter* studied (*11*, *24*). A close relationship of the isolates to these bacteria is suggested. Accordingly, their characteristics were compared with the bacteria (*1*, *11*, *25*, *26*). The isolates were definitely distinguished from these Q-10-equipped strains with respect to the phenotypic characteristics (Table 1) and the cellular fatty acid composition (Table 3) (*27*). Only the isolates required ethanol for growth and did not form dihydroxyacetone from glycerol or form 5-ketogluconic acid (Table 1).

The isolates are distinguished from the members of the genus *Acetobacter*, i.e., *A. liquefaciens* IAM 1834^T, *A. xylinum* NCIB 11664^T and IFO 3288, and *A. hansenii* NCIB 8746^T. They were negative in oxidation of ethanol and lactate to CO_2 and H_2O, in their reaction in acetate and lactate, and in oxygen consumption for acetate and succinate (Table 1).

Furthermore, the isolates are different from *Gluconobacter* strains, *G. cerinus* IFO 3267^T, *G. oxydans* subsp. *oxydans* ATCC 19357^T, *G. oxydans* subsp. *suboxydans* IFO 3990, *G. oxydans* subsp. *industrius* IFO 3260, *G. oxydans* subsp. *melanogenes* IFO 3293 and *G. oxydans* subsp. *sphaericus* IFO 12467^T in containing a straight-chain $C_{14:0}$ acid in their cellular fatty acids and in the negative result for ferric chloride reaction in fructose.

The isoprenoid quinone and cellular fatty acids apparently distinguish the isolates from *Frateuria aurantia* which has Q-8 (Q-7) as major ubiquinones (*8*, *11*) and the branched-chain fatty acid, *i*-$C_{15:0}$ acid as the major component of cellular fatty acids (*8*, *27*). Isoprenoid quinone analyses also distinguish the isolates from *A. aceti*, *A. pasteurianus* and *A. peroxydans* which have Q-9 or Q-9 (Q-8) as major ubiquinones (*11*, *28*).

Recently, several reports suggesting reconsideration of several classifications in Bergey's Manual of Determinative Bacteriology, 8th ed. (*17*) were published. GOSSELÉ et al. (*22*) proposed that the single species *G. oxydans* should not be further divided into subspecies. YAMADA (*25*) proposed treating *A. aceti* subsp. *xylinum* as a species of *Acetobacter*, *A. xylinum*. GOSSELÉ et al. (*23*) and YAMADA et al. (*28*) proposed classifying *A. aceti* subsp. *liquefaciens* as *A. liquefaciens* as

Table 5. Characteristics differentiating *A. polyoxogenes* sp. nov. from

Characteristic	*A. polyoxogenes* sp. nov.	*A. liquefaciens* (Asai 1935) Gosselé et al. 1983
Oxidation of ethanol to CO_2 and H_2O	−[c]	+[a,d]
Oxidation of lactate to CO_2 and H_2O	−	+[d]
Reaction in: acetate	−	+
lactate	−	+
Oxygen consumption for: acetate	−	+
succinate	−	+
Formation of: 5-ketogluconic acid	−	d[b,d]
2,5-diketogluconic acid	−	+[d]
Dihydroxyacetone from glycerol	−	+[d]
Ferric chloride reaction in: glucose	−	d[d]
fructose	−	+[d]
Formation of cellulose	−	−
Formation of brown pigment	−	+[d]
Growth on: Frateur's modified Hoyer medium-ethanol (vitamin added)	−	+[d]
Growth in the presence of 10% acetic acid	+	−
Growth at pH 4	−	+
Growth on: medium not containing acetic acid	−	+
medium not containing ethanol	−	+
Ubiquinone system	Q-10	Q-10 [Q-9][g]
Cellular fatty acid type	18: 1, 14: 0	18: 1, 14: 0[h]
Guanine plus cytosine content of DNA (mol%)	57.6–58.1	62.3–64.6[i]

[a] 90% or more of the strains are positive.
[b] 11–89% of the strains are positive.
[c] 90% or more of the strains are negative.
[d] GOSSELÉ et al. (*23*).
[e] YAMADA et al. (*24*, *25*).

an independent species in the genus *Acetobacter*. GOSSELÉ et al. (*23*) proposed a new species, *A. hansenii* including some strains of *A. aceti* subsp. *xylinum*, *A. aceti* subsp. *orleanensis* and *A. pasteurianus* subsp. *pasteurianus* and this species was described in Bergey's Manual of Systematic Bacteriology (*19*) published recently. However, they did not consider the isoprenoid quinone as a taxonomic criterion in their study (*23*). Therefore, strains with Q-10 or with Q-9 were included in a species (*26*). It seemed likely that the taxonomic concept of *A. hansenii* should be reconsidered since isoprenoid quinone is understood to be a useful and significant criterion for classifying various microorganisms (*29*, *30*).

With the above information, DNA-DNA homologies were studied to assess the genetic relationship between the isolates and some authentic acetic acid bacteria. Four strains of the isolates had extremely high indexes of DNA homology among themselves, whereas the authentic strains showed low homology indexes to the isolated strains. In the authentic strains, the strains of *A. xylium* had

other species of acetic acid bacteria with Q-10 or G-10 [Q-9].

A. xylinum (ex Brown 1886) Yamada 1983	*G. oxydans* (Henneberg 1897) De Ley 1961	*G. cerinus* (ex Asai 1935) Yamada and Akita 1984
+[e]	−[f]	−[f]
+[e]	−[f]	−[f]
+[e]	−	−
+[e]	−	−
+	−	−
+	−	−
+[e]	+[f]	+[f]
−	d[f]	−[f]
d[e]	+[f]	+[f]
−[e]	d[f]	−[f]
d[e]	d[f]	d[f]
d[e]	−	−
−[e]	d[f]	−[f]
d[e]	−[f]	−[f]
−	−	−
+	+	+
+	+	+
+	+	+
Q-10[g]	Q-10[g]	Q-10[g]
18:1; 14:0[h]	18:1[h]	18:1[h]
54.9–62.8[i]	58.1–62.8[j]	54.2–57.6[j]

[f] Gosselé et al. (*22*).
[g] Yamada et al. (*24*, *25*).
[h] Yamada et al. (*27*).
[i] Gillis and De Ley (*21*).
[j] Yamada et al. (*31*).

relatively high homology indexes to the isolated strains. Strains of *G. cerinus*, which was recently proposed by Yamada and Akita (*31*), were not included in our DNA-DNA hybridization experiments. However, it is apparent that the isolates differ from *G. cerinus* in their phenotypic features (Table 1). Recent studies of DNA homologies among *Gluconobacter* strains revealed that they were divided into two or more groups according to one study (*32*) and at least three groups according to another study (*33*). They also showed that the homology indexes among the groups were very low. The results indicated that *Gluconobacter* was a genetically diverse genus, while De Ley and Swings (*20*) regarded it as a genus with a single species.

From these results, we conclude that the isolates should be classified as a new species belonging to the genus *Acetobacter* rather than the genus *Gluconobacter*, giving more importance to the chemotaxonomic properties: The isolates contained the straight-chain $C_{14:0}$ acid, which is characteristic of the cellular fatty acid of the genus *Acetobacter* (*27*), and the index of the DNA homology between the isolate

NBI 1028 and *A. xylium* NBI 1002 was 48%, while the phenotypic properties indicated negative results for oxidation of ethanol and latate to CO_2 and H_2O, reaction in acetate and lactate, and oxygen consumption for acetate and succinate. Recently, YAMADA and KONDO (*34*) proposed *Gluconoacetobacter*, a new subgenus for the Q-10-equipped, acetate-oxidizing acetic acid bacteria. The isolates are tentatively classified in the subgenus *Gluconoacetobacter*, although they did not oxidize acetate. Based on the above results, we propose a new species *Acetobacter polyoxogens* sp. nov. for the isolates.

Description of Acetobacter polyoxogenes sp. nov.

po. ly. o. xo' ge. nes. Gr. adj. *poly* much; Gr. n. *oxos* vinegar; Gr. v. *gennaio* produce; M. L. adj. *polyoxogenes* producing much vinegar.

Gram-negative rods, occurring singly or in pairs, 0.4 to 0.6 μm wide by 0.8 to 1.3 μm long. Non-motile. Forms glossy, smooth, light grey to light brown and small punctiform colonies. Grows on AE agar plate in an atomosphere with a relative humidity of 95 to 100% and in AE broth with shaking. Grows at pH 2.2 to 3.5 and at 15 to 35°C. Requires more than 4% acetic acid for growth. Does not grow on medium without ethanol or glucose. Does not grow on Frateur's modified Hoyer medium (vitamin added). Produces gluconic acid and 2-ketogluconic acid from glucose, and acetic acid from ethanol. Does not produce 5-ketogluconic acid, γ-pyrones, water soluble brown pigment or cellulose from glucose. Produces neither γ-pyrones from fructose nor dihydroxyacetone from glycerol. Does not oxidize lactate, acetate, or succinate. Utilizes D-glucose, D-fructose, sucrose, D-sorbitol, D-mannitol, ethanol, and *n*-propanol. Utilization of *iso*-propanol is variable. Possesses Q-10 as a major isoprenoid quinone. The DNA base composition ranges from 57.6 to 58.1 mol% guanine plus cytosine. The cellular fatty acids are of the straight-chain type containing $C_{18:0}$ as a major component.

Habitat: Fermented vinegar broth with high acidity.

The type strain: NBI 1060 (JCM 3808).

Strains examined: NBI 1028, NBI 1029, NBI 1036, NBI 1037, NBI 1038, NBI 1039, NBI 1041, NBI 1042, NBI 1043, NBI 1044, NBI 1045, NBI 1057, NBI 1058, and NBI 1060.

Table 5 shows the characteristics differentiating the new species from other species with Q-10 or Q-10 (Q-9) in the genera *Acetobacter* and *Gluconobacter*.

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A. polyoxogenes, the new species proposed here, was treated under another name, *A. altoacetigenes*, in our previous paper (*1*) before it was effectively described. The name has been changed because the species epithet "*altoacetigenes*" was a Latin and Greek hybrid (Recommendation 6, International Code of Nomenclature of Bacteria (*35*)).

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