Journal of Microbiology and Biotechnology
Vol. 3, No. 2, 108-114, 1993

Characterization of a New Acidophilic *Acetobacter* sp. Strain HA Isolated from Korean Traditional Fermented Vinegar

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A new strain of acidophilic, acetogenic bacterium, *Acetobacter* sp. strain HA was isolated by selective enrichment from the traditionally fermented rice wine vinegar in Korea. It was a gram-negative, non-motile short rod and oxidized acetate and lactate. The optimal temperature and pH for growth were 28°C and 4.0, respectively. The strain HA differed from other *Acetobacter* species by growing well on methanol, xylitol, inositol, dulcitol, D-xylose, L-arabinose, and D-mannose as sole sources of carbon and energy. The isolated strain HA did not produce γ-pyrones from glucose and did not produce ketone bodies from glycerol. The quinone system used in this study was an ubiquinone-9 isoprene unit. The guanine-plus-cytosine content of the DNA was 50.7 mol%, and the major cellular fatty acids were $C_{18:1}$ and $C_{16:0}$.

Acetic acid bacteria can use ethanol as an energy source, and they have acid-tolerance. Classical sources of acetic acid bacteria were fermented liquors and vinegars, and one of the pioneers was Takahashi (16) who had worked on Japanese sake vinegar fermentations. Acetic acid bacteria are now classified into two genera, *Acetobacter* and *Gluconobacter* based on the preferential oxidation of carbon sources. The ability of *Acetobacter* to oxidizing lactate and acetate (2, 6) as well as ethanol (3) are metabolic attributes that have been exploited in the past. De Ley (5) also implied that preferential carbohydrate utilization might be used to differentiate *Acetobacter* and *Gluconobacter*. Recent investigations for the classification of acetic acid bacteria have a tendency to use new genetic techniques, such as cellular fatty acid composition, ubiquinone system, and DNA G+C content analysis. In 1981, Yamada *et al.* (21) reported that the cellular fatty acid composition in acetic acid bacteria gave a criterion for the differentiation of *Gluconobacter* and *Acetobacter*. Also, the ubiquinone system was applied for subgrouping microorganisms such as *Acetobacter xylinum* with Q-10, *Acetobacter aceti*, and *Acetobacter pasteurianus* with Q-9 (20, 22, 23).

Little work has been done for the taxonomic analysis of the acetic acid bacteria the habitants in Korean traditional fermented vinegar so far. In order to preserve the gene pool and to develop new materials, we have isolated *Acetobacter* sp. from the traditional vinegar microflora. There are four species of *Acetobacter* currently recognized according to Bergey's Manual of Systematic Bacteriology (6). But the possibility of finding out some novel species of *Acetobacter* or new types of microorganism has not been excluded. Thus, this work deals with the characteristics of acidophilic, acetogenic bacterium which was isolated from Korean traditional fermented rice wine vinegar, and the improvement of the Korean type strains in acetic acid production.

We successfully isolated a bacterial strain from the Korean rice wine fermented vinegar which proved to be a gram-negative, acidophilic methanol-utilizing, and short-rod-shaped organism that grows aerobically in the acidic pH range: this strain was named as *Acetobacter* sp. strain HA. In this work, we propose the taxonomic position of the strain HA which we isolated from a rice wine vinegar for the first time.

MATERIALS AND METHODS

Bacterial Strains and Media

The strain HA was isolated from vinegar. Traditionally fermented rice wine vinegar samples were collected from southern areas in Korea. These samples were plated onto a medium containing 1% yeast extract, 3% ethanol, 5% acetic acid, and 2.5% agar (7). The plates were incu-

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Key words: Acetic acid bacteria, *Acetobacter*, chemotaxonomy

bated at 28℃ for 3 days and the colonies were transferred on to a new plate. This procedure was repeated until a pure culture was obtained. In addition, the HA strain was grown and primarily identified as *Acetobacter* on DSM agar: 10 g of proteose peptone, 3 g of yeast extract, 15 g of calcium lactate, 1 g of dextrose, 1 g D-sorbitol, 2 g D-mannitol, 1 g monopotassium phosphate, 0.02 g manganese sulfate monohydrate, 0.03 g bromocresol purple, 0.004 g cycloheximide, 0.1 g sodium desoxycholate or 29.5 micrograms of brilliant green, and 15 g of agar per liter (pH 4.0).

Acetobacter aceti ATCC 23746, *Acetobacter methanolicus* ATCC 43581, and *Acetobacter pasteurianus* ATCC 9428 were obtained from the American Type Culture Collection (Rockville, Maryland, USA). These strains were grown and maintained on a GYC agar (1% yeast extract, 3% glucose, 1% $CaCO_3$, and 2% agar). The temperature for growth was 28℃; these strains were maintained at 2 to 4℃ and were transferred to new media every 4 weeks.

Morphological, Physiological and Biochemical Tests

The gram staining was carried out by Hucker's modification method, and the flagella were stained by the Loeffler method (8, 11).

The Carr's method (3) was used to detect the overoxidation of ethanol or lactate to CO_2 and H_2O. The γ-pyrone compounds were detected with ferric chloride as a developing agent (1). The formation of pigments was tested on a GYC medium (9). The utilization of organic substrates as energy sources was tested in the medium containing 0.05% yeast extract. The compounds were dissolved in the medium, sterilized passing through 0.2 μm sterile syringe filters (Gelman Sciences, Ann Arbor, MI, USA) or autoclaved depending on their heat stabilities, and added to the medium to make the following final concentration: aromatic acids, 10 mM; methanol, 6.25 or 62.5 mM; and all others at 0.5%. The medium of Uhlig et al. (18) was used to detect acid production from different carbon compounds; the tests were finished after 7 days of incubation at 28℃. The tests developed by Swings *et al.* (15) were used to determine the L-amino acid utilization.

More biochemical tests were performed using an API 20E kit (Bio : Meriux, France) and an Oxi/Ferm tube system (F. Hoffman-La Roche, Switzerland). Their Resistance to antibiotics was tested using the method of Lennette (12).

Cellular Fatty acid Composition

The cellular fatty acids were extracted by the method of Yamada *et al.* (21). The methyl esters of the cellular fatty acids were analyzed on a Shimadzu GC 14A Model gas chromatography equipped with a hydrogen flame ionization detector. The temperature at injection and detection was 205℃, and the column temperature was retained at 185℃. Nitrogen gas was used as carrier gas at a flow rate of 30 ml per min. Fatty acids were identified by comparing the relative retention time with those of their methyl esters. The composition of the fatty acids was calculated from the ratio of their peak areas to the total area.

Mol Percent Guanine-Plus-Cytosine

The DNA was isolated as described by Marmur (13). The cells were lysed by treatment with EDTA and lysozyme, and the DNA was treated with nuclease P1 and bacterial alkaline phosphatase. The mol percent of guanine + cytosine was determined by a high-performance liquid chromatography (Shimadzu HPLC system, Japan) (17). The nucleosides were eluted with a mixture of 0.6 M $NH_4H_2PO_4$ (pH 4.0) and acetonitrile (20 : 1, vol/vol) at a flow rate of 1 ml/min at room temperature. Each nucleoside was monitored at 270 nm.

Purification and Identification of the Quinone

The ubiquinones were extracted and purified according to the method of Yamada *et al.* (20). Crude lipid was extracted from lyophilized cells by shaking vigorously in ether-ethanol mixture (2 : 1, vol/vol) for 1 h at room temperature. The combined extract was evaporated, and the residue was dissolved in a small amount of acetone. The resulting solution was subjected to a thin-layer chromatography on a silica gel plate (HPTLC-Fertigplatten, Merck, USA) for purification. The loaded samples were developed by using a mixture of acetone and water (4 : 1, vol/vol). Chromatograms were identified under ultraviolet light after 15 minutes of development.

RESULTS AND DISCUSSION

Cells of strain HA were gram negative, non-motile, and short rod-shaped (about 0.5×1.1 μm in size) and they occurred singly or sometimes in pairs. They did not form endospores. The optimum growth temperature of the strain HA was between 28 and 30℃. The optimum pH was around 4.0 and the isolated strain was able to grow on 10% acetic acid. Also, they have tolerance to ethanol up to 20% and were able to grow at initial pH as low as 2.5. We detected no growth at pH 8.0 and below 2.0 (not shown in Table 1). Because of their abilities to oxidize ethanol to acetate and glucose to gluconate in acidic media, and to form acids from several sugars and alcohols like many other acetic bacteria (10, 18), we designated the isolated strain as a new acidophilic *Acetobacter* sp. strain HA.

On dextrose sorbitol mannitol (DSM) agar, the strain HA was primarily identified to be belonging to the genus *Acetobacter*. The strain HA changed its color from yel-

Table 1. Morphological and biochemical characteristics of the isolated strain HA.

Characteristics	Result	Characteristics	Result
Colony form	Circular	Substrate utilization[b]	
Cell arrangement	Single	Glucose	+
Shape	Short rod	Mannitol	+
Gram stain	−	Inositol	+
Motility	−	Sorbitol	+
VP test	+	Rhamnose	+
Gelatin liquefaction	−	Sucrose	+
Nitrate reduction	−	D-Mannose	+
Catalase reduction	+	Amygdalin	+
Oxidase test	−	L-Arabinose	+
β-galactosidase	−	Methanol	+
Arginine dehydrolase	−	*n*-Propanol	+
Lysine decarboxylase	−	*n*-Butanol	+
Citrate utilization	−	Allyl alcohol[c]	−
H_2S production	−	Xylitol	+
Urease test	−	Dulcitol	+
Tryptophan deaminase	+	D-Xylose	+
Indole production	−	Resistance to antibiotics	
Growth on acetic acid 10%	+	Kanamycin	1 μg
Growth on single L-amino acids[a]		Streptomycin	2 μg
as sole source of nitrogen		Tetracycline	4 μg
L-Glutamine	+	Neomycin	32 μg
L-Glutamic acid	+	Chloramphenicol	32 μg
L-Proline	+	Ampicillin	256 μg
L-Alanine	+	Nalidixic acid	512 μg

[a] L-amino acids tested but not utilized: arginine, asparagine, aspartic acid, cysteine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine.

[b] Substrates tested but not utilized: D,L-arabitol, D-lyxose, melibiose, L-sorbose, cellobiose, maltose, raffinose, gluconate, 2 ceto-gluconate, 5 ceto-gluconate, L-fucose, D-tagatose, glycogen, inuline, saccharose, maltose, erythritol, arbutin, salicine, esculin, melezitose, *n*-octanol, isoamylalcohol, mercaptoethanol, and D-fructose.

[c] Strain HA was affected by lower allyl alcohol than 1mM when tested MIC on complex agar medium containing increasing cencentrations of allyl alcohol.

Abbreviations: +, positive or utilized; −, negative or not used

low to purple on DSM agar which was devised to provide differentiation of *Gluconobacter* and *Acetobacter* based on the preferential oxidation of carbon sources (4). Selectivity was achieved by acidification and the incorporation of cycloheximide to inhibit yeast and mold growth. To minimize any possible interference by other acid tolerant gram positive bacteria, 0.1 g of sodium desoxycholate per liter of medium was added.

Gluconobacter, unable to oxidize lactate, preferentially oxidizes the minor carbohydrate constituents producing acetic acid and maintains the yellow color of the medium. But, *Acetobacter* grown on DSM changed the color from yellow to purple and produced a white precipitate of calcium carbonate. Precipitated calcium carbonate contiguous with cell growth often forms a translucent halo zone (3).

Table 1 shows some characteristics of the strain HA. The isolate was not able to do gelatin liquefaction and to form indole and H_2S. The strain HA showed positive reaction in VP, catalase reduction, tryptophan deaminase tests, and it utilized L-glutamine, L-glutamic acid, L-proline, and L-alanine as sole sources of nitrogen. Experiments dealing with the utilization of more than 40 carbon compounds (e.g., sugars, sugar alcohols, organic acids, and their salts) as sole sources of carbon gave the results described in Table 1. The strain HA cells showed sensitivity to allyl alcohol. Since allyl alcohol kills only cells exhibiting alcohol dehydrogenase (ADH) activity (14, 24), it was proposed that the strain HA might have ADH activity.

In antibiotic resistance tests, the strain HA was considered to be strongly resistant to ampicillin (256 μg) and nalidixic acid (512 μg), but was susceptible to kanamycin (1 μg), streptomycin (2 μg), and tetracycline (4 μg).

Table 2 compares some properties of the strain HA with those of the genera *Gluconobacter* and *Acetobac-*

Table 2. Characteristics of *Gluconobacter* and *Acetobacter* compared with those of the isolated strain HA[a].

Characteristics	*Gluconobacter*	*Acetobacter*	Strain HA
Flagellation of mobile strains:			
Polar	+	−	−
Peritrichous	−	+	−
Overoxidation of ethanol	−	+	+
Overoxidation of DL-lactate to CO_2 and H_2O	−	+	+
Oxidation of acetate to CO_2 and H_2O	−	+	+
Ketogenesis from glycerol	+	D	−
Formation of brown water-soluble pigments on GYC agar	−	D	−
Product formed from D-glucose:			
2-Ketogluconic acid	+	D	−
5-Ketogluconic acid	+	−	−
2,5-Diketogluconic acid	D	D	−
Type of Ubiquinone formed			
Q9	−	D	+
Q10	+	D	−
Acid produced from:			
Inositol	D	−	−
Maltose	D	−	−
D-Fructose	+	−	(+)
Carbon sources for growth:			
Acetate	−	D	+
Lactate	−	D	+
Guanine-plus-cytosine content of DNA (mol%)	57-64	51-65	50.7

[a] Data for *Gluconobacter* and *Acetobacter* from reference 18.
Abbreviations: +, positive; (+), weakly positive; −, negative; D, different reactions in different taxa.

ter, as given by De Ley *et al.* (6). From these results, the newly isolated strain HA was clearly identified as a member of the genus *Acetobacter*. The strain tested showed weak acid production from D-fructose, but not from maltose or sorbitol.

This strain had the ability to reduce catalase, and it produced CO_2 from acetic and lactic acid, but it did not produce ketone bodies from glycerol. Also, the strain HA converted the glucose into gluconate, but it was not able to oxidize gluconate to either 2-ketogluconate or 5-ketogluconate. Thus it is considered to belong to the oxydans group of *Acetobacter* as reported by De Ley (5).

To investigate the relationship between strain HA and the genus *Acetobacter* in detail, the quinone systems and the cellular fatty acid composition were examined. Test results suggest that the quinone of strain HA is ubiquinone-9. Almost all of the *Acetobacter* species have Q-9 system, but *Gluconobacter* species and *Acetobacter xylinum* have Q-10 system (21). Q-8 system was found in the polarly flagellated intermediate strains of acetic acid bacteria (19).

As shown in Table 3, the strain HA had relatively

Table 3. The cellular fatty acid composition in the genus *Acetobacter* and *Gluconobacter* compared with those of the strain HA[a].

Species and strain number	Fatty acid[b] (%)			
	14:0	16:0	18:0	18:1
G. gluconicus IFO 3171	−	16.4	6.4	59.2
G. melanogenus IFO 3293	−	13.6	2.3	69.5
G. oxydans IFO 3189	−	15.4	2.7	66.5
A. aceti IFO 3281	3.0	21.2	8.8	62.3
A. pasteurianus IFO 3223	1.0	11.7	8.7	61.5
A. rancens IFO 3191	2.3	12.5	6.3	62.7
A. xylinus subsp. *xylinus* IFO 3288	4.4	10.9	2.4	76.3
A. xylinus subsp. *liquefaciens*				
IFO 12388	7.5	15 9	6.3	56.6
IFO 12257	5.9	11.1	6.8	57.3
Strain HA	1.7	22.0	0.7	72.1

[a] All data except the data strain HA obtained from reference 21.
[b] The abbreviations for the fatty acids are: 14 : 0, a straight-chain saturated acid of 14 carbon atoms; 16:0, a straight-chain saturated acid of 16 carbon atoms; 18 : 0, a straight-chain saturated acid of 18 carbon atoms; 18 : 1, a straight-chain unsaturated acid of 18 carbon atoms with one double bond.

simple cellular fatty acid profiles. The main fatty acids of strain HA were straight-chain saturated $C_{16:0}$ acid (22 %) and straight-chain unsaturated $C_{18:1}$ acid (72.1%). The minor fatty acids identified (less than 10% of the total acids) were $C_{14:0}$, $C_{18:0}$, and 2-OH $C_{14:0}$ acids. This composition is the same as the compositions found in genus *Acetobacter* species that have fatty acid patterns comprised mainly of $C_{16:0}$, $C_{18:1}$, and minor $C_{14:0}$ acid which can not be observed in genus *Gluconobacter*.

Characteristics which differentiate the species among the genus *Acetobacter* (15) were compared with the those of the strain HA (Table 4). It is obvious that the strain HA is different from any other *Acetobacter* species. This result is matching well with the protein gel eclectrophoresis patterns of HA cell extracts when compared with those of other extracts from strains of *Acetobacter*: *A. aceti*, *A. methanolicus*, *A. pasteurianus*, or *A. xylinum* (data not shown here). The strain HA had 50.7 mol% of DNA G+C content. This was close to the lower limit of the G+C contents found in the members of the genus *Acetobacter*. The strain HA is also different from other *Acetobacter* species based on the utilization of methanol. dulcitol, sorbitol, xylitol, D-xylose, D-mannose, and L-arabinose as carbon sources. Some features not shown in Table 2 and 4 are as follows: the strain HA formed acid from glycerol, xylose, adonitol, rhamnose, dulcitol, melibiose, raffinose, *n*-propanol, *i*-butanol, *n*-butanol, and methanol. Although the strain HA utilized methanol, it was totally different from methylotrophic bacteria, *A. methanolicus* based on its quinone systems and DNA G+C content (Table 4).

The newly isolated acidophilic *Acetobacter* sp. strain HA described above is physiologically and biochemically very similar to organisms which belong to the genus *Acetobacter* (6). Within the genus *Acetobacter* it is not possible to assign the strain HA to any previously publi-

Table 4. Characteristics that differentiate the species of the genus *Acetobacter* and the strain HA.

Characteristics	*A. aceti* ATCC 23746	*A. liquefaciens*[a]	*A. pasteurianus* ATCC 9428	*A. methanolicus* ATCC 43581	HA strain
Formation of:					
water-soluble brown pigment on GYC agar	−	+	−	−	−
γ-Pyrones from D-glucose	−	d	−	−	−
γ-Pyrones from D-fructose	−	+	−	−	−
5-ketogluconic acid from D-glucose	+	d	−	−	−
2,5-Diketogluconic acid from D-glucose	−	+	−	−	−
Ketogenesis from glycerol	+	+	−	(+)	−
Growth on carbon sources:					
Methanol	−	−	−	+	+
Ethanol	+	+	+	(+)	+
Dulcitol	−	−	−	−	+
Sorbitol	−	d	−	−	+
Glucose	+	+	+	+	+
Sodium acetate	+	d	+	(+)	+
Growth on L-amino acids in the presence of D-mannitol as a carbon source:					
Glycine,threonine,tryptophan	−	d	−	−	−
L-glutamine	−	+	−	−	+
Growth in the presence of 10% ethanol	−	−	−	−	+
Ubiquinone type (major part)	Q_9	Q_{10}	Q_9	Q_{10}	Q_9
G+C content (mol%)	55.9	62.3-64.6	52.8	62.3	50.7

[a] Data obtained from reference 6.

Abbreviations: +, positive; (+), weakly positive; d, positive in 11 to 89% of the strains; −, negative.

shed species. As described above, the strain HA has its own unique characteristics as compared to *A. aceti*, *A. pasteurianus*, *A. liquefaciens*, and *A. methanolicus* (Table 4). Sequencing of the 5S and 16S rRNAs is being carried out to clarify the phylogenetic or evolutionary position of *Acetobacter* sp. strain HA.

Descriptions of *Acetobacter* sp. Strain HA

The cells are short rods that occur singly or in pairs, Gram-negative, non-sporulating, and non-motile. The optimum temperature for growth is about 28℃. The optimum pH for growth is around 4.0 and it has a characteristics of catalase positive, VP test positive, methanol utilization, resistance to 256 μg ampicillin and 512 μg nalidixic acid.

The G+C content of the DNA is 50.7 mol% (as determined by HPLC).

Straight-chain saturated $C_{16:0}$ and a straight-chain unsaturated $C_{18:1}$ acid are the major components of the cellular fatty acids. Ubiquinone-9 is the major component of the quinone system.

Acetobacter sp. strain HA was isolated from the rice wine vinegar fermented in the traditional method in Haenam, the southern part of Korea.

Acknowledgement

The Present Study was Supported by the Basic Science research Institute Program, Ministry of Education, Republic of Korea, 1992, Project No. BSRI-92-424.

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(Received April 17, 1993)