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Isolation and Characterization of *Acetobacter* sp. CS Strains from Haenam Vinegar

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Two strains of the gram-negative acetic acid bacteria, *Acetobacter* sp. strain CS2 and CS5, were isolated from the traditional raw rice wine vinegar of Haenam area. The strains oxidized ethanol to acetic acid and over-oxidized acetate and lactate to CO_2 and H_2O. They produced 2-ketogluconic acid from glucose but did not produce γ-pyrones from glucose and dihydroxyacetone from glycerol. The CS strains possessed ubiquinone-9 as a major isoprenoid quinone and contained straight-chain $C_{18:1}$, $C_{16:0}$, and $C_{14:0}$ fatty acids. The DNA base composition of the CS2 and CS5 strains was 56.2 and 57.3 mole% G+C, respectively. The isolates were grown well on methanol, gluconate, erythritol, raffinose, dulcitol and xylitol as sole sources of carbon and energy which are different from those of other *Acetobacter* species and produced acid from sucrose, glycerol, fructose, inositol, mannitol, and ribose.

KEY WORDS □ ***Acetobacter*, acetic acid fermentation, characteristics, taxonomy**

Acetic acid bacteria are well known as vinegar-making organisms, the name "acetic acid bacteria" being derived from vinegar. These bacteria are now classified into two genera, *Acetobacter* and *Gluconobacter*, both of which have a strong ability to oxidize sugars and alcohols (15). Well-known examples are the production of acetic acid from ethanol and the oxidation of glucose to gluconic acid.

In spite of the high technology involved in industrial vinegar fermentation today and the immense production figures, only little is known about the bacteriology of this process (11). Although special attention has recently been paid on these organisms (10, 16), the biochemical and genetic aspects of the acetic acid bacteria have not been so extensively studied as alcohol brewing yeasts, lactic acid bacteria, and most extensively studied *Escherichia coli*. The low level of knowledge is mainly due to the difficulties in the isolation and cultivation of organisms producing acetic acid actively (11). Thus high acid vinegar fermentations yielding 12 to 13% final acetic acid concentrations are performed under non-sterile conditions with a spontaneously developing and largely uncharacterized acetobacters.

There have been many reports that morphological and physiological characteristics of acetic acid bacteria are variable. They include the ability of cellulose formation, gluconic acid production, ketogenesis, pigmentation, *etc.* (6, 14). Furthermore, the strains which lost the ability to oxidize ethanol, a representative characteristics of acetic acid bacteria, also have been reported (14). In this respect it is important to know more about the bacteriology of these organisms and the biochemistry of ethanol oxidation. In addition, isolation and characterization of the strains responsible for acetic acid production in vinegar sources are essential to obtain the genetic insight as well as for breeding improved strains of this organism.

In this paper, we define the characteristics of and discuss the taxonomic position of methanol-utilizing acetic acid bacteria which have been isolated from Haenam vinegar.

MATERIALS AND METHODS

Isolation of acetic acid bacteria

The acetic acid bacteria were isolated from the traditional raw rice wine vinegar produced in the Haenam area. Two kinds of media, i.e., AE medium (1% yeast extract, 3% ethanol, 5% acetic acid) and modified Carr medium (1% yeast extract, 3% ethanol, 0.0022% bromocresol-green, pH 6.8), were employed for the isolation (5). At first, the samples were spread on AE agar plates and incubated at 30°C for 3 days. Colonies appeared on the AE agar plates were picked up and purified by repeated streak culture on the same medium. The purified colonies were

streaked onto modified Carr medium. The resulting pure culture was then grown on GYC agar slants (1% yeast extract, 3% glucose, 1% $CaCO_3$, 2% agar) for 2 days at 30°C and stored at 4°C. The stock culture was transfered to fresh GYC slants bi-monthly.

Bacterial strains

In addition to the isolates mentioned above, the following strains were used for comparison: *Acetobacter aceti* ATCC 23746, *Acetobacter pasteurianus* ATCC 9428, *Acetobacter methanolicus* ATCC 43581.

Morphological characteristics

Cell shape was observed with an optical microscope using cells stained with carbolfuchsin. Gram stain was carried out by Hucker's modification (4). Motility was observed by the method of Mac Faddin (12).

Physiological and biochemical characteristics

Effect of pH and acetic acid on growth of the isolates was examined by the method of Entani *et al.* (5). Ethanol, salt, and glucose tolerance were examined as described by De Ley *et al.* (3). The method of Carr (2) was used to detect the overoxidation of ethanol and the oxidation of lactate to CO_2 and H_2O. Utilization of various carbon compounds was tested by the cultivating the isolates in the media containing 0.05% yeast extract and an appropriate carbon source. The carbon sources were sterilized separately by filtration and added to the sterile media to a final concentration of 0.3~2%. 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 30°C. Growth on methanol was studied using methanol-containing basal medium (or agar) and Hoyer medium as modified by Frateur (3), using methanol concentrations of 0.3~1% as a sole source of carbon and energy as described by Gossele *et al.* (8). Ketogenesis from glycerol and the formation of γ-pyrones from glucose and fructose were determined by using the methods of Yamada *et al.* (19). Formation of pigments was tested on GYC medium (8). Utilization of NH_4^+ or NO_3^- as a sole nitrogen source was studied in the presence of methanol, ethanol, and mannitol in Frateur's modified Hoyer ethanol-vitamins medium (3). Formation of 2-ketogluconic acid from glucose was tested in gluconate peptone broth (12). After incubation for 2 days, 1 m*l* of Benedict's solution was added directly to the medium and boiled for 10 min. An appearance of orange-red precipitates was interpreted as a positive result. The biochemical tests of catalase, oxidase, citrate utilization, hydrolysis of urea and lactose, arginine dihydrolase, formation of H_2S and indole, formation of acetyl-methylcarbinol from sodium lactate (VP test), nitrate reduction, and gelatin liquefaction were carried out by using API 20E Kit (bio:Meriux, France).

Isoprenoid quinones

The isolates were cultivated in AE broth for 5 days at 30°C with shaking. Ubiquinones were extracted and purified according to the method of Yamada *et al.* (20). The extracts were subjected to thin-layer chromatography (HPTLC-Fertigplatten, Merck). The loaded samples were developed using a mixture of acetone and water (80:20). After development for 15 min, chromatograms were examined under ultraviolet light.

Cellular fatty acid composition

The cellular fatty acids of the isolates were extracted by the method of Yamada *et al.* (21). Methyl esters of the cellular fatty acids were analyzed using a Shimadzu GC 14A gas-chromatography (Kyoto, Japan) equipped with a hydrogen flame ionization detector. The temperature at injection and detection ports was 205 °C. Column temperature was retained at 185°C. Nitrogen gas was used as a carrier at a flow rate of 30 m*l* per min.

Determination of base composition

DNA was isolated and purified by using procedure of Marmur (13). The average guanine-plus-cytosine (G+C) contents of the nuclease P1 and bacterial alkaline phosphatase treated DNA of the isolates were determined by high-performance liquid chromatography (Shimadzu HPLC system) (17). Nucleosides were eluted by a mixture of 0.6 M $NH_4H_2PO_4$ (pH 4.0) and acetonitrile (20:1, v/v) at a flow rate of 1 m*l*/min at room temperature. Each nucleoside was detected by absorbance at 270 nm.

RESULTS AND DISCUSSION

Two strains of acetic acid bacteria, CS2 and CS5, were isolated from Haenam vinegar. The isolates were gram-negative non-motile rods. The cell size of the isolates was approximately 0.6 μm×1.2 μm. The size of the colonies grown on GYC for 3 days at 30°C was about 1 mm in diameter. The colonies were slightly convex, circular with entire margin, and light grey in color.

The two isolates showed a pronounced acid tolerance and were able to grow on at initial pH as low as 2.5. The optimal pH was found to be pH 5.5 for both strains. The number of the isolates increased from 3.3×10^7 cells/m*l* to 3.2×10^9 cells/m*l* in 3 days at optimal pH. No growth was occured at pH 8.0 and below 2.0. They produced acetic acid from ethanol in acidic pH, and further oxidized acetate and lactate to CO_2 and H_2O. The isolates were found to have catalase activity. The strains produced acetyl-methylcarbinol from sodium acetate and 2-ketogluconic acid from glucose. They reacted negatively in the following tests: dihydroxyacetone production from

Table 1. *Characteristics of CS strains compared with those of Gluconobacter and Acetobacter.*

Characteristcs	*Gluconobacter*[a]	*Acetobacter*[a]	CS strains
Overoxidation of ethanol	−	+	+
Oxidation of lactate and acetate to CO_2 and H_2O	−	+	+
Ketogenesis	+	D	−
Formation of brown water-soluble pigments on GYC agar	−	D	−
Cellular fatty acids type	18:1	18:1, 14:0	18:1, 14:0
Type of Ubiquinone formed			
Q9	−	D	+
Q10	+	D	−
Carbon sources for growth			
Acetate	−	D	+
Lactate	−	D	+

[a] Data for *Gluconobacter* and *Acetobacter* from De Ley *et al.* (3).
D, Different reactions among different taxa.

Table 2. *Tolerance of CS strains to acetic acid, ethanol, salt, and glucose.*

Growth on	CS2	CS5
Acetic acid		
5%	+	+
7%	+	+
9%	+	+
12%	w	+
Ethanol		
5%	+	+
10%	+	+
15%	+	+
NaCl		
2%	+	+
3%	+	w
Glucose		
15%	+	+
20%	+	+
25%	w	−
30%	−	−

W, Weakly positive.

glycerol, formation of γ-pyrones from glucose and fructose, formation of brown pigment, oxidase, nitrate reduction, formation of H_2S and indole, citrate utilization, hydrolysis of urea and lactose, arginine dihydrolase, and gelatine liquefaction.

Table 1 compares some chracteristics of the CS strains with characteristics of the genera *Gluconobacter* and *Acetobacter,* as given by De Ley *et al.* (3). These chracteristics show obvious conformity with the genus *Acetobacter.*

Ammonium and nitrate were utilized as sole sources of nitrogen, but amino acids were not. On mannitol or ethanol as a sole carbon source they did not require vitamins as growth factors but they required at least 0.05% yeast extract.

The results of acetic acid, ethanol, salt, and glucose tolerance tests are shown in Table 2.

Experiments dealing with the utilization of more than 30 carbon compounds as sole sources of carbon gave the results below. None of the strains grew on formaldehyde, iso-amylalcohol, starch, cellobiose, cellulose, rhamnose, and sodium benzoate. They grew on methanol, ethanol, iso-propanol, n-butanol, dulcitol, sorbitol, inositol, xylitol, mannitol, ribose, fucose, gluconate, arabinose, sorbose, maltose, raffinose, Na-acetate, Na-lactate, glycerol, erythritol, succinic acid, Na-pyruvate, glucose, fructose, and sucrose. The CS strains produced acids during growth on glucose, maltose, mannose, erythritol, mannitol, iso-propanol, n-butanol, methanol, sucrose, fructose, glycerol, ribose, inositol, and dulcitol.

A ubiquinone homologue with nine isoprene units (Q-9) was the major component of isoprenoid quinone in the cells of the CS strains and with eight isoprene units (Q-8) as the minor component. They contained large amounts of unsaturated fatty acid with straight chain ($C_{18:1}$). Small amounts of straight-chain saturated $C_{16:0}$ acid, and $C_{14:0}$ acid were also found in the strains.

The base composition of the DNA of the two strains, CS2 and CS5, was 56.2 and 57.3 mol% G+C, respectively.

Because of the features described above, acidophilic, methanol-utilizing bacterial strains CS2 and CS5 clearly belong to the genus *Acetobacter* (3). This conclusion is supported by the over-oxidation of acetic acid, and small amounts of $C_{14:0}$ straight-chain saturated fatty acid. This fatty acid was detected in addition to the $C_{18:1}$ straight-chain unsaturated fatty acid. As described by Yamada *et al.* (21), the latter (as a major component of celluar fatty acids) is a typical fatty acid of acetic acid bacteria, and the former is that of *Acetobacter.* Within the genus *Acetobacter,* the following four species were described in Bergey's Manual of Systematic Bacteriology, vol. 1 (3): *Acetobacter aceti, A. liquefaciens, A. pasteurianus,* and *A. hansenii.* Since then, the following additional proposals have been published: the revival of *Acetobacter xylinum* (22), the creation of a new subgenus *Gluconoacetobacter* within the genus *Acetobacter* to contain the acetobacters with ubiquinone Q-10 (23), and the descriptions of the new species; *Acetobacter polyoxogenes* (5), *A. methanolicus* (18), and *A. diazotrophicus* (7).

Characteristics which clearly differentiate the CS strains from *A. aceti, A. pasteurianus, A. liquefaciens, A. methanolicus A. hansenii* and *A. diazotrophicus* are listed in Table 3.

Table 3. *Characteristics that differentiate several species of the genus Acetobacter and the CS strains.*

Characteristics	CS strains	*A. aceti*[a]	*A. liquefaciens*[a]	*A. pasterianus*[a]	*A. hansenii*[a]	*A. methanolicus*[a,b]	*A. diazotrophicus*[b]
Formation of:							
Water-soluble brown pigments on GYC agar	−	−	+	−	−	−	+
γ-Pyrones from D-Glucose	−	−	±	−	−	−	+
γ-Pyrones from D-Fructose	−	−	+	−	−	−	+
2-Ketogluconic acid from glucose	+	+	+	±	+	−	+
Ketogenesis from glycerol	−	+	+	−	−	−	±
Voges-Proskauer test	−	±	±	±	±	−	ND
Growth on the following carbon sources:							
Methanol	+	−	−	−	−	+	−
Erythritol	+	±	+	−	−	−	ND
Dulcitol	+	−	−	−	±	−	ND
Xylitol	+	−	±	−	−	ND	ND
Raffinose	+	−	−	−	±	−	ND
Acid produced from							
Fructose	+	−	−	−	−	−	ND
Inositol	+	−	−	−	−	ND	ND
Mannitol	+	−	−	−	−	±	ND
Growth on 10 % ethanol	+	−	−	+	−	−	−
Ubiquinone type	Q9	Q9	Q10	Q9	ND	Q10	Q10
Guanine plus Cytosine content of DNA (mol%)	56.2 and 57.3	56-60	62-65	53-63	58-63	62	61-63

[a]Data from De Ley *et al.* (3), Gillis *et al.* (7) and this study; [b]Data from Gillis *et al.* (7).
±, 11 to 89% of the strains are positive; ND, not determined.

The CS strains were clearly distinguished from the methanol-utilizing acetic acid bacteria, *A. methanolicus*, with respect to the phenotypic characteristics, DNA base composition, and the ubiquinone system. Also more detailed investigations resulted in finding considerable differences between the CS strains and *A. pasteurianus* which is shared some common features with the CS strains such as ubiquinone system, ketogenesis from glycerol and high ethanol resistance.

As the most important differentiating characteristic, the utilization of methanol is discussed first. The oxidation of methanol by strains of *A. pasteurianus* has been reported previously by Gossele *et al.* (9). Methanol is fixed by means of hexulose phosphate synthase, and incorporate into cells via the fructose biphosphate variant of the hexulose phosphate pathway (1). Babel (1) commented in explanation that phosphofructokinase is the key enzyme for assimilation of methanol in the acetic acid bacteria strains which he tested. But Babel did not detect phosphofructokinase in the methanol-utilizing *A. pasteurianus* strains of Gossele (1). In addition, Uhlig *et al.* (18) found that these strains did not grow on methanol as a sole carbon source on agar and in liquid media. However, The CS strains grew well in the yeast basal medium containing methanol and on Frateur's modified Hoyer vitamin medium supplemented with methanol in place of ethanol.

Additional differnces between the CS strains and *A. pasteurianus* include good growth of the CS strains on gluconate, raffinose, xylitol, erythritol and dulcitol, and acid formation of the CS strains on fructose, mannitol, inositol and glycerol. Moreover, in contrast to *A. pasteurianus*, the CS strains gave a negative Voges-Proskauer reaction with lactate and produced 2-ketogluconic acid from glucose.

As discussed above, the CS strains exhibit properties which have not been described previously for *Acetobacter*. From these results, we conclude that the CS strains may be considered as separate species or subspecies of the genus *Acetobacter*.

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초 록: 해남식초에서의 *Acetobacter* sp. CS 균주의 분리 및 특성

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해남지방의 전통적인 막걸리 식초로부터 분리된 초산 발효 균주 CS2, CS5는 에탄올로부터 초산을 생성하고 이 초산을 재산화시키므로 *Acetobacter*로 확인되었다. 분리 균주들은 glucose로부터 2-ketogluconic acid를 형성하였고 15%의 ethanol과 12%의 acetic acid 농도에서도 자라는 높은 저항성을 보여주었다. Ubiquinone system으로 Q-9을 함유하고 DNA base composition은 각각 56.2와 57.3 mole% G+C이었다. CS 균주들은 methanol, gluconate, dulcitol, raffinose, xylitol을 탄소 및 에너지원으로 이용하고 fructose, glycerol, mannitol, ribose 등으로부터 산을 형성하는 점에서 기존의 *Acetobacter*들과는 많은 차이점을 보여 새로운 species로 추정할 수 있었다.