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Communication

Production of 4-Keto-D-arabonate by Oxidative Fermentation with Newly Isolated *Gluconacetobacter liquefaciens*

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Production of 4-keto-D-arabonate (4KAB) was confirmed in a culture medium of *Gluconacetobacter liquefaciens* strains, newly isolated from water kefir in Argentina. The strains rapidly oxidized D-glucose, D-gluconate (GA), and 2-keto-D-gluconate (2KGA), and accumulated 2,5-diketo-D-gluconate (25DKA) exclusively before reaching the stationary phase. 25DKA was in turn converted to 4KAB, and 4KAB remained stable in the culture medium. The occurrence of 4KAB was assumed by Ameyama and Kondo about 50 years ago in their study on the carbohydrate metabolism of acetic acid bacteria (*Bull. Agr. Chem. Soc. Jpn.*, 22, 271–272, 380–386 (1958)). This is the first report confirming microbial production of 4KAB.

Key words: acetic acid bacteria; *Gluconacetobacter liquefaciens*; 4-keto-D-arabonate; oxidative fermentation

In oxidative fermentation by acetic acid bacteria, D-glucose is oxidized sequentially to D-gluconate (GA) by quinoprotein D-glucose dehydrogenase (GDH). GA is further oxidized to 2-keto-D-gluconate (2KGA) by flavoprotein GA dehydrogenase (GADH), and then to 2,5-diketo-D-gluconate (25DKA) by 2KGA dehydrogenase (2KGDH) (Fig. 1).[1)] GA oxidation to 5-keto-D-gluconate (5KGA) is catalyzed by quinoprotein glycerol dehydrogenase.[2)] All the enzymes involved in the formation of GA and various ketogluconates are membrane-bound, and the catalytic sites face the periplasmic space.[1)] These metabolic intermediates are important products utilized in various chemical and pharmaceutical industries, including L-ascorbate (vitamin C) production. 25DKA figures in the production of 2-keto-L-gulonate, an intermediate in the Reichstein synthesis of L-ascorbate,[3–7)] which is formed stereospecifically by NADPH-dependent 25DKA reductase.

In contrast with the industrial significance of 2-keto-L-gulonate, sporadic studies of 25DKA degradation were done but not followed up extensively. Recently, we isolated several strains of acetic acid bacteria from the Argentine water kefir. Four strains among them accumulated 25DKA remarkably in the culture medium in 2 d of cultivation. The isolates were examined for phenotypic characteristics by methods reported by Asai *et al.*,[8)] Yamada *et al.*,[9)] and Gosselé *et al.*[10)] The cells were ellipsoidal to rod-shaped, approximately 0.8–1.0 × 1–3 μm in size, and occurred singly, in pairs or in short chains. They were strictly aerobic, gram-negative, and catalase-positive. Colonies are smooth, round, convex, white, and opaque. They produce brown water-soluble pigments. Acetate and lactate are oxidized. All the isolates grew weakly or slowly in the presence of 30% D-glucose, and they grew on D-mannitol and L-glutamate agar. Dioxyacetone was produced from glycerol. Acid was produced from D-arabitol, L-arabinose, *meso*-erythritol, D-fructose, D-glucose, glycerol, D-mannose, and D-xylose, but not from L-arabitol, dulcitol, D-galactose, lactose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, ribitol, D-sorbitol, L-sorbose, or sucrose. Their phenotypic characteristics were close to those of *Ga. liquefaciens* NBRC 12388^T (Table 1).

To the best of our knowledge, after sequential oxidation of D-glucose or GA to 2KGA, 25DKA accumulates in the culture medium as an oxidation product of 2KGA, catalyzed by membrane-bound 2KGDH (Fig. 1).[1,11)] The four isolated strains rapidly accumulated 25DKA when cultivated in a medium containing 0.5% D-glucose, 2.0% Na-gluconate, 0.3% glycerol, 0.3% yeast extract (Oriental Yeast, Tokyo), and 0.2% polypeptone. 25DKA in culture broth of the four isolated strains was in turn converted to an unidentified compound when cultivation was prolonged to the late stationary phase, irrespective to the presence

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Abbreviations: GA, D-gluconate; 4KAB, 4-keto-D-arabonate; 2KGA, 2-keto-D-gluconate; 5KGA, 5-keto-D-gluconate; 25DKA, 2,5-diketo-D-gluconate

Table 1. Phenotypic Characteristics of Isolates and *Gluconacetobacter liquefaciens* NBRC 12388^T and *Ga. sacchari* LMG 19747^T

Characteristic	RCTMR 9	RCTMR 10	RCTMR 11	RCTMR 12	*Ga. liquefaciens*	*Ga. sacchari*
Oxidation of acetate and lactate	+	+	+	+	+	+
Dioxyacetone from glycerol	+	+	+	+	+	+
Growth on mannitol agar	+	+	+	+	+	+
Growth on glutamate agar	+	+	+	+	+	+
Growth on 30% Glucose	S	S	S	S	+	+
Growth at 37°C	−	−	−	−	−	−
Acid production from						
D-Arabitol	+	+	+	+	+	−
L-Arabitol	−	−	−	−	−	−
L-Arabinose	+	+	+	+	+	+
Ducitol	−	−	−	−	−	−
meso-Erythritol	+	+	+	+	+	−
D-Fructose	+	+	+	+	+	+
D-Galactose	−	−	−	−	+	+
D-Glucose	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+
Lactose	−	−	−	−	−	−
Maltose	−	−	−	−	−	−
D-Mannose	+	+	+	+	+	+
D-Mannitol	−	−	−	−	−	−
Melibiose	−	−	−	−	+	−
Raffinose	−	−	−	−	−	−
L-Rhamnose	−	−	−	−	−	−
Ribitol (adonitol)	−	−	−	−	w	−
D-Sorbitol	−	−	−	−	−	−
L-Sorbose	−	−	−	−	−	−
Sucrose	−	−	−	−	+	−
D-Xylose	+	+	+	+	+	+

+, positive; w, weak positive; −, negative; S, grew slowly.
Results for 3–5 d of incubation.

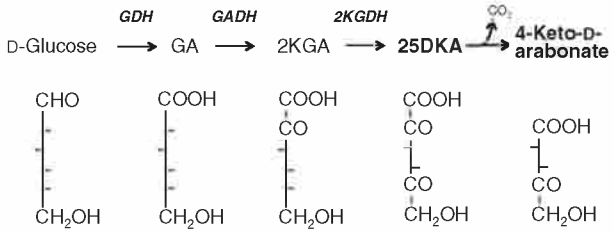

Fig. 1. D-Glucose Oxidizing Systems in Acetic Acid Bacteria Leading to the Formation of 4-Keto-D-arabonate.

The enzymatic steps focused on in this paper are shown in bold. GDH, quinoprotein D-glucose dehydrogenase; GADH, FAD-dependent D-gluconate dehydrogenase; 2KGDH, FAD-dependent 2-keto-D-gluconate dehydrogenase.

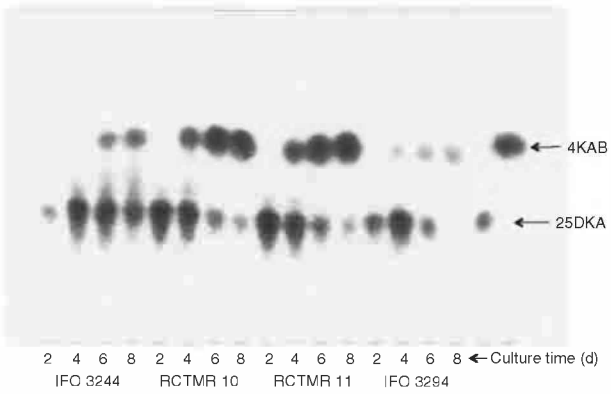

Fig. 2. TLC Chromatogram of 4-Keto-D-arabonate Formation by *Gluconacetobacter liquefaciens*.

Ga. liquefaciens RCTMR 10 and RCTMR 11, *G. oxydans* IFO 3244, and *G. melanogenus* IFO 3294 were grown in the medium, and an aliquot of the culture medium was taken at 2-d intervals, as indicated by the numbers along the base line of the chromatogram. TLC chromatography was done with a solvent of *t*-butanol:formic acid:H_2O = 4:1:1.5. The developed plate was sprayed with TTC solution.

or absence of $CaCO_3$ in the culture medium. 25DKA and the unidentified compound were reactive to 2,3,5-triphenyltetrazolium chloride (TTC), giving deep red spots on a TLC chromatogram.[12] Judging from the intensity of the spots, more than 80% of 25DKA appeared to be converted to the unidentified compound when examined with strains RCTMR 10 and RCTMR 11 and two standard 25DKA-producing strains (Fig. 2). The cultivation profiles and the chromatographic behaviors of strains RCTMR 9 and RCTMR 12 were the same as those of strains RCTMR 10 and RCTMR 11. As reference strains, *Gluconobacter subxoydans* IFO 12528, *G. dioxyacetonicus* IFO 3271, and *G. melanogenus* IFO 3294 were used as the standard 5KGA, 2KGA, and 25DKA producing strains respectively.[13] *G. oxydans* IFO 3244 was used as a reference strain, by which 2KGA was first formed. Then it was converted to 25DKA and only a part of 25DKA was converted to the unidentified compound. *G. melanogenus* IFO 3294 accumulated 25DKA, but not to the unidentified compound.

After removal of the cells by centrifugation of the culture broth (300 ml) of a 6-d culture of *Ga. liquefaciens* strain RCTMR 10, the supernatant was adsorbed into a Dowex 1 × 4 column (acetate form, 1.5 × 25 cm). After the column was washed with water and then with 0.5 M acetic acid, a gradient elution of acetic acid up to

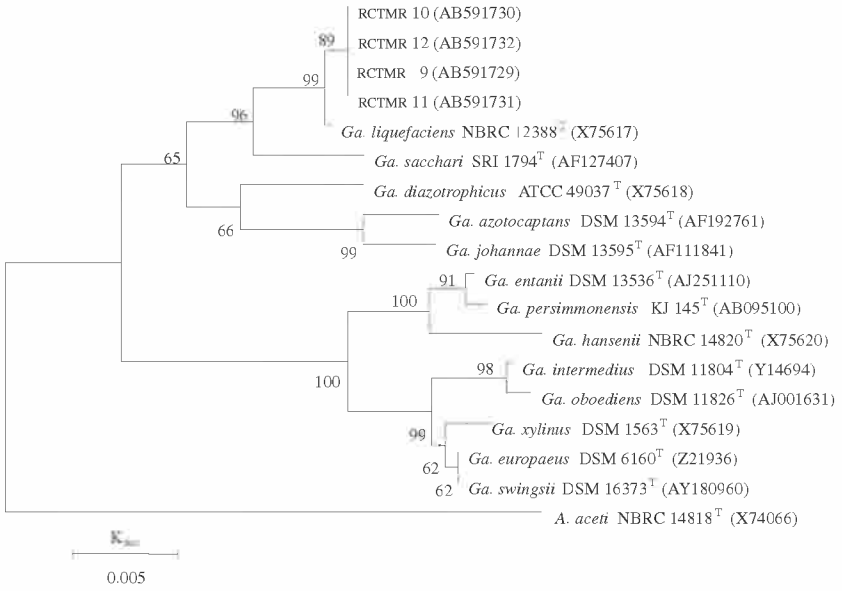

Fig. 3. Phylogenetic Tree of 16S rRNA Gene Sequencing of Isolated Strains RCTMR 9, RCTMR 10, RCTMR 11, and RCTMR 12.

The 16S rDNA sequences of the four isolates were determined as described by Yukphan *et al.*[16,17] Multiple sequence alignments were made with the program CLUSTAL X (version 1.81).[18] Alignment gaps and ambiguous bases were excluded from calculations of similarity. Sequence comparisons were made for 1,420 bases (positions 28–1,472 by the *Escherichia coli* numbering system, accession no. V00348)[19] of the 16S rRNA gene. Distance matrices were calculated by the two-parameter method of Kimura (K_{nuc}).[20] Phylogenetic trees were constructed by the neighbor-joining method.[21] Robustness of individual branches was estimated by bootstrapping with 1,000 replications.[22] Bootstrap values below 50% are not shown. Pair-wise sequence similarities were calculated.

2.5 M was done. The unidentified compound gave a single spot with TTC on a TLC plate, indicating that it was free of impurities. The acetic acid in the pooled fractions was removed by evaporation under reduced pressure at 50 °C. The resulting viscous liquid was neutralized with fine powdered $CaCO_3$. After the brown color was removed by the addition of a small amount of activated charcoal, the solution was gradually evaporated until crystals appeared.

The white crystals of the unidentified compound were analyzed, HR-ESI-MS (negative mode) gave 163.0240 $(M-1)^-$, corresponding to a molecular formula of $C_5H_8O_6$. ^{1}H-NMR showed four signals at 4.46 (d, J = 2.0 Hz), 4.57 (s), 4.63 (s), and 4.69 (d, J = 2.0 Hz). The ^{13}C-NMR and DEPT showed five carbons; three of them were alcoholic at 65.99, 73.07, 76.88. There was one carboxylic carbon at 177.13, and one signal of ketone carbon at 212.38. The HMBC cross peak showed correlations of H-5/C-4, C-3, and H-2/C-1. Judging from these data, the isolated compound was identified as 4-keto-D-arabonate (4KAB = 4-pentulosonic acid) (Fig. 1). Other data, including IR and 2D-NMR such as COSY and HMQC, also confirmed this structure. 4KAB was a compound of which the occurrence was postulated by Ameyama and Kondo in 1958.[14,15] There has been no report or information about 4KAB formation in oxidative fermentation as well as in other fields of natural sciences. Thus, there is little information about 4KAB, although 4KAB production by acetic acid bacteria has become available.

The results of phylogenetic identification of the isolated strain, *Ga. liquefaciens*, according to 16S RNA gene sequencing, showed 99.8% identity with the type strain, *Ga. liquefaciens* IFO 12388 (Fig. 3). The four strains showed 100% similarity to each other. In our previous study,[23] *Ga. liquefaciens* IFO 12388, *Ga. liquefaciens* IFO 12257, *G. sphaericus* IFO 12467, and *G. oxydans* IFO 3293 showed 25DKA accumulation, but no formation of a new spot corresponding to 4KAB was observed. Type strain *Ga. liquefaciens* IFO 12388 did not form 4KAB, although the four isolated strains were identical to the type strain at 99.8% identity. The isolated strains were finally identified as *Ga. liquefaciens*, and were registered as strains RCTMR 9, RCTMR 10, RCTMR 11, and RCTMR 12 at the Research Center for Thermotolerant Microbial Resources (RCTMR) at Yamaguchi University.

In a forthcoming study, structural identification of a novel oxidative decarboxylase catalyzing 4KAB formation from 25DKA will be done to determine whether the enzyme contains two functions in one enzyme, or whether it is an enzyme complex including decarboxylase and sugar oxidase activities.

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