Facultatively Anaerobic Bacteria Showing High Productivities of 2,5-Diketo-D-gluconate from D-Glucose

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Forty-two bacterial strains that produce calcium 2,5-diketo-D-gluconate from 30% (w/v) D-glucose with yields of more than 70 mol% were isolated from various soil and fruit samples. They were small rod-shaped, gram-negative, non-spore-forming and facultatively anaerobic bacteria, and could be classified taxonomically into three groups. Representative strains (SHS 2003, ATCC 31623; SHS 2006, ATCC 31626; SHS 2008, ATCC 31628) of the latter were identified as members of the genus *Erwinia*. The DNA base ratios of these strains ranged from 46.3 to 48.7 mol% guanine plus cytosine.

The results of experiments using whole cells and cell extracts of these three strains suggested that D-glucose is oxidized to 2,5-diketo-D-gluconate via D-gluconate and 2-keto-D-gluconate. No 5-keto-D-gluconate was detected in the culture broth throughout their cultivation. Most activities of enzyme(s) responsible for the oxidation of D-glucose to 2,5-diketo-D-gluconate were found in the particulate fractions of these strains.

Calcium 2,5-diketo-D-gluconate (Ca-25DKG; the free acid being referred to as 25DKG herein) is biochemically converted to calcium 2-keto-L-gulonate,¹⁾ which is a key intermediate in vitamin C synthesis.²⁾

Since Katznelson *et al.*³⁾ first prepared and identified 25DKG as a fermentation product of *Acetobacter melanogenus*, the ability of microorganisms to produce 25DKG from D-glucose has been reported for aerobic bacteria, *i.e.*, several strains of *Acetobacter*^{2~4)} and *Gluconobacter*,⁵⁾ and *Acetomonas alboesamae*⁶⁾ (*Pseudomonas alboesamae* Wakisaka 1964⁷⁾). A strain of *A. alboesamae* has effectively produced Ca-25DKG from D-glucose. This strain, however, could not tolerate concentrations of D-glucose higher than 20% (w/v) and required a fermentation time of about 60 hr. 25DKG is unstable, especially as to heat and alkaline conditions.³⁾ A short fermentation time is important for preventing the decomposition of 25DKG produced. Because of this, we searched for the most efficient strains, as to Ca-25DKG production, found in

nature. Consequently, we isolated 42 strains that produced Ca-25DKG from 30% (w/v) D-glucose with excellent yields, *i.e.*, of more than 70 mol%, within 40 hr in 1-l fermentors. All these isolates were facultatively anaerobic bacteria and were classified into the genus *Erwinia*. One of them was further improved through mutagenic treatment, finally producing 328.6 mg of Ca-25DKG per ml with a 94.5 mol% yield from D-glucose on 26-hr cultivation, as reported previously.¹⁾

This paper describes a taxonomic survey of the facultatively anaerobic isolates showing such high productivities of Ca-25DKG from D-glucose, and their metabolic pathway for 25DKG production.

MATERIALS AND METHODS

Chemicals. Ca-25DKG and calcium 5-keto-D-gluconate were prepared by the methods previously described.¹⁾ Calcium 2-keto-D-gluconate and sodium D-gluconate were purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). Other chemicals used for analyses were of reagent grade. All components of the media used were commercial

preparations, unless otherwise noted.

Isolation and cultivation of 25DKG-producing strains. Soil, fruit and vegetable samples were dispersed in sterile water, respectively, and then plated onto medium A, which contained 1 g of D-glucose, 0.1 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.1 g of peptone (Difco), 0.1 g of KH_2PO_4 , 0.02 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7 g of CaCO_3 and 2 g of agar in 100 ml of tap water. The colonies with clear zones around them due to the dissolution of CaCO_3 were isolated and transferred to stock slants (GYP slants), which contained 0.5 g of glycerol, 0.5 g of yeast extract (Difco), 0.3 g of peptone (Difco), 0.1 g of KH_2PO_4 , 0.02 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2 g of agar in 100 ml of tap water. The isolates grown on GYP slants were streaked onto agar plates of medium B, which contained 10 g of D-glucose, 1.5 g of corn steep liquor, 0.1 g of KH_2PO_4 , 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.9 g of CaCO_3 and 2 g of agar in 100 ml of tap water. The pHs of media A, B and GYP were adjusted to 7.0 before sterilization at 121°C for 20 min. Each of the strains grown on medium B agar plates was then inoculated with a loop of cells taken from a stock slant into medium C (50 ml in a 500-ml baffled flask), which contained 25 g of D-glucose, 1 g of corn steep liquor, 0.1 g of KH_2PO_4 , 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 7.2 g of CaCO_3 in 100 ml of tap water, and then incubated at 28°C for 48 hr on a rotary shaker at 250 rpm. The pH of medium C was adjusted to 7.0 before sterilization at 121°C for 20 min. Each of the 25DKG-producing strains that was confirmed to produce Ca-25DKG in medium C as described under Analytical methods was inoculated again from a GYP slant culture into the seed medium (50 ml in a 500-ml flask), which contained 1 g of D-glucose, 5 g of corn steep liquor, 0.1 g of KH_2PO_4 , 0.02 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of NaCl and 0.5 g of CaCO_3 in 100 ml of tap water. The seed culture was shaken at 28°C for 14 hr on a rotary shaker at 250 rpm.

The fermentor medium (medium D) contained 11.8 g of D-glucose, 3.5 g of corn steep liquor, 0.12 g of KH_2PO_4 and 9.7 g of CaCO_3 in 100 ml of tap water. Each 1-l fermentor (280 ml of medium D) was inoculated with 50 ml of a seed culture, followed by incubation at 28°C with an air flow rate of 1 vvm and agitation at 1760 rpm. A sterile solution of 50% (w/w) D-glucose was fed twice to the culture as described under RESULTS. Each fermentation was continued for 49 hr. The pHs of the seed and fermentation media were adjusted to 7.0 before sterilization at 121°C for 20 min. Bacterial growth was measured as the change in optical density at 660 nm with a Hitachi spectrophotometer, model 139.

Analytical methods. D-Glucose, D-gluconate, 2-keto-D-gluconate, 25DKG and 5-keto-D-gluconate were identified by paper chromatography, according to the method described by Wakisaka,⁸⁾ and also by gas chromatography.¹⁾ The concentrations of Ca-25DKG and calcium 2-keto-D-

gluconate were determined by the $\text{NH}_4\text{OH-HCl}$ method as described previously.¹⁾ D-Glucose was measured enzymatically by the method of Mollering and Bergmeyer.⁹⁾ D-Glucose was also determined enzymatically by the Glucose-B-Test (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Identification procedures. 25DKG-Producing strains were taxonomically characterized by conventional methods for morphological, biochemical and physiological properties.¹⁰⁻¹³⁾ Morphological characteristics were observed over a period of 24 hr for cultures grown on bouillon agar and mannitol agar¹⁴⁾ at 28°C. Flagellation was observed electron-microscopically by the method of Hillier *et al.*¹⁵⁾ with a Nihon Densi transmission electron microscope, model JEM 100 B. Anaerobic growth of 25DKG-producing strains on GYP agar plates and mannitol agar plates¹⁴⁾ was examined at 28°C for 72 hr in a Gas-pack anaerobic system (BBL, Dickson & Co., Cockeysville, U.S.A.) with *Pseudomonas aeruginosa* and *Escherichia coli* as controls. The utilization of organic compounds, production of reducing substances from sucrose, NaCl tolerance, and hydrolysis of casein and cotton seed oil were examined by the methods of Dye.¹⁶⁾ The decarboxylation of amino acids was examined by the method of Shaw and Clark.¹⁷⁾ Pigment production and mucoid growth were observed under the conditions described by Lelliott and Dickey.¹⁸⁾ Pectate degradation and deoxyribonuclease (DNase) activity were examined on Paton's pectate gel medium¹⁹⁾ and Bacto DNase agar (Difco), respectively. Ubiquinone was extracted and determined by the method described by Yamada *et al.*²⁰⁾ The guanine plus cytosine (G+C) content of the deoxyribonucleic acid was estimated from the thermal denaturation temperature (T_m) and the buoyant density in CsCl by the methods of Marmur and Doty,²¹⁾ and Schidraut *et al.*,²²⁾ respectively.

Preparation of cell extracts, soluble fractions and particulate fractions. Cell were grown aerobically at 28°C for 6 hr in a medium containing 5 g of D-glucose, 0.5 g of peptone (Difco), 0.5 g of yeast extract (Difco) and 2.0 g of CaCO_3 in 100 ml of tap water. Cells were harvested by centrifugation ($12,000 \times g$ for 10 min at 4°C) and then washed twice with 0.1 M phosphate buffer (pH 6.0). The cell paste was suspended in the same fresh buffer and then disrupted by passage through a French pressure cell press at 1500 kg/cm². Cell debris and unbroken cells were removed by centrifugation ($15,000 \times g$ for 10 min at 4°C). The resulting cell extract was further centrifuged ($156,000 \times g$ for 120 min at 4°C), which yielded particulate and soluble fractions. The particulate material was resuspended in fresh 0.1 M phosphate buffer (pH 6.0). The soluble fraction used for the product analyses was dialyzed overnight at about 4°C against 0.05 M phosphate buffer (pH 7.0).

Reaction products of D-glucose, D-gluconate, 2-keto-D-gluconate and 5-keto-D-gluconate with the cell extracts. Reaction products were measured with two reaction mixtures, I and II. Reaction mixture I consisted of 10 μmol of substrate, 50 μmol of 2,6-dichlorophenolindophenol (DCPIP), 20 μmol of phenazine methosulfate (PMS), 150 μmol of phosphate (pH 6.0) and the cell extract (0.5 mg of protein) in a total volume of 3.0 ml. Reaction mixture II contained 50 μmol of substrate, 50 μmol of NAD (P)⁺, 2.5 μmol of KCN, 125 μmol of phosphate (pH 7.0) and the dialyzed soluble fraction (10–12 mg of protein) in a total volume of 2.5 ml. The reaction was started by adding the substrate and then the mixture was allowed to stand at 28°C. The reaction time was 30 min for mixture I and 2 hr for mixture II, respectively. The reaction products were determined by the methods described under *Analytical methods*.

Enzyme assays. The reaction mixture contained 0.5 μmol of 2,6-dichlorophenolindophenol, 0.2 μmol of phenazine methosulfate, 100 μmol of substrate (D-glucose, sodium D-gluconate or sodium 2-keto-D-gluconate), 100 μmol of phosphate (pH 6.0) and the soluble or particulate fraction in a total volume of 3.0 ml. Enzyme activities were assayed spectrophotometrically at 600 nm with a Hitachi spectrophotometer, model 200-10.

One unit of enzyme activities was defined as the amount of enzyme that reduced 1 μmol of 2,6-dichlorophenolindophenol per minute at 28°C. The extinction coefficient of 2,6-dichlorophenolindophenol was 11 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ at pH 6.0. Protein concentrations were determined by the method of Lowry *et al.*²³⁾ with bovine serum albumin as standard.

RESULTS

Isolation of 25DKG-producing strains

After a suitable incubation time of 2 to 4 days, 369 strains of acid-forming bacteria were first isolated from 235 soil, vegetable and fruit samples on agar plates containing 1% (w/v) D-glucose (medium A). Acid formation was confirmed by the appearance of clear zones around the growing colonies on the agar plates containing CaCO_3 . The ability of these isolates to tolerate high sugar concentrations was tested on agar plates containing 10% (w/v) D-glucose (medium B). Then, the 203 strains that grew on the above agar plates (medium B) were cultivated in baffled flasks containing 25% (w/v) D-glucose (medium C), and 96 strains were confirmed to have accumulated more or less Ca-25DKG in the final broth by paper chromatography.

The Ca-25DKG productivity of each of the 96 strains thus selected was further tested in a 1-l fermentor containing medium D. At 6-hr and 12-hr cultivation, 99 and 165 g of a sterile solution of 50% (w/w) D-glucose were fed to the culture, respectively. The total amounts of D-glucose added to the culture were equivalent to 30% (w/v) of the initial concentration in the medium D. Under these conditions, 42 strains could convert D-glucose to Ca-25DKG with yields of more than 70 mol% within 40-hr cultivation. After 30-hr cultivation, three representative strains, SHS 2003, SHS 2006 and SHS 2008, had accumulated 278 mg, 285 mg and 256 mg of Ca-25DKG per ml, respectively. The yields of 25DKG from D-glucose were 76 mol% for strain SHS 2003, 78 mol% for strain SHS 2006 and 70 mol% for strain 2008, respectively. In the culture broth of all 42 strains, the formation of calcium D-gluconate and calcium 2-keto-D-gluconate, as intermediates, was detected on paper chromatography, whereas no calcium 5-keto-D-gluconate was detected throughout their cultivation.

Taxonomic studies on the 25DKG-producing strains

All the 42 strains that showed high Ca-25DKG productivities were small (0.8 to 1.5 by 1.0 to 3.3 μm in size), gram-negative, non-spore-forming and facultatively anaerobic bacteria, occurring singly or in pairs. They grew weakly in a Gas-pack system and paraffin-stab cultures. On the basis of the results of various taxonomical tests, the 42 strains were classified into three groups, represented by strains SHS 2003, SHS 2006 and SHS 2008. The differences in taxonomical properties among the three groups were mainly in cell size, motility, decarboxylation of glutamate, reaction on litmus milk, production of reduced compounds from sucrose, Voges-Proskauer reaction, acid production from mannitol, glycerol, lactose and sucrose, utilization of acetate and lactate, G+C content, ubiquinone, temperature for growth, and pH for growth (Table I). The common charac-

TABLE I. TAXONOMICAL DIFFERENCES AMONG THE THREE REPRESENTATIVE STRAINS, SHS 2003, SHS 2006 AND SHS 2008

Parameters	SHS 2003 (ATCC 31623)	SHS 2006 (ATCC 31626)	SHS 2008 (ATCC 31628)
Cell size (μm):			
Diameter	0.8~1.2	1.1~1.3	0.8~0.9
Length	1.0~2.9	1.3~1.9	1.5~1.7
Motility	—	—	+
Flagella	None	None	Lateral
Ubiquinone	8 and 7	8	8 and 7
Acidification and coagulation of Litmus milk	+	—	—
Decarboxylation of L-glutamate	—	+	—
Hydrolysis of lactose	+	—	—
Voges-Proskauer reaction	w	w	+
Production of reducing compounds from sucrose	—	+	+
Temperature for growth ($^{\circ}\text{C}$)	8~39	6~39	8~40
Temperature for optimum growth ($^{\circ}\text{C}$)	18~31	21~28	20~33
pH for growth	5.5~8.0	5.5~8.5	5.5~7.5
Acid production from:			
Mannitol and lactose	+	—	—
Glycerol	+	—	+
Sucrose	—	+	+
Utilization of:			
Acetate and lactate	+	$\pm$	+
G+C contents (mol%):			
Bouyant density	45.8	47.8	49.1
T_m	46.3	48.5	48.7
Origin	Mandarin orange	Mandarin orange	Soil

Symbols and abbreviations: +, positive; —, negative; $\pm$, negative or slightly positive; w, weakly positive.

teristics of the three representative strains are as follows: oxidation-fermentation test, facultative; methylene blue reduction, positive; catalase, positive; oxidase, negative; KCN inhibition, positive; nitrite produced from nitrate; nitrate respiration, negative; urease not produced; casein not hydrolyzed; gelatin not liquefied; hydrogen sulfide not produced; ammonia not produced; decarboxylation of L-lysine, L-arginine and L-ornithine, negative; starch not hydrolyzed; citrates utilized; methyl red test, positive; mucoid growth on sucrose medium observed; ethanol not oxidized to acetic acid; grew in 5% NaCl; nicotinamide required for growth; lipase, negative; cotton seed oil not hydrolyzed; pectate not degraded; deoxyribonuclease activity, negative; no formation of a blue, pink, yellow or fluorescent pigment; acid but no gas from fruc-

tose, glucose, galactose, β -methyl glucoside, mannose, ribose, salicin, xylose, melibiose and cellobiose; no acid and no gas from maltose, dextrin, esculin, rhamnose, sorbitol, raffinose, adonitol, dulcitol, melezitose, arabinose, α -methyl glucoside or inositol; citrate, formate, fumarate, gluconate, succinate and malate utilized; and benzoate, galacturonate, malonate, oxalate, propionate and tartrate not utilized.

The three representative strains, SHS 2003, SHS 2006 and SHS 2008, have many characters in common with species belonging to the family *Enterobacteriaceae*, as described by Brenner²⁴: small, gram-negative, motile (with lateral flagella or flagellum) or non-motile, and facultatively anaerobic rods; metabolism, respiratory and fermentative; catalase, positive; oxidase, negative; nitrite produced from ni-

trate; and G + C content, 38 ~ 60 mol%. In addition, they share some basic properties with species of the genus *Erwinia*, as described by Lelliott and Dickey¹⁸⁾: acid production from β -methylglucoside and sucrose, positive (strain SHS 2003 did not produce acid from sucrose but utilized sucrose); acid production from adonitol, dulcitol and melezitose, rarely; benzoate, oxalate and propionate not utilized; gas production, comparatively weak or absent; starch not hydrolyzed; decarboxylase for glutamate, negative (strain SHS 2006 was positive); urease or lipase production, rarely; temperature for growth, below 40°C. On the basis of the above similarities, we classified the three representative strains, SHS 2003, SHS 2006 and SHS 2008, as members of the genus *Erwinia*, although there, at present, seems to be several unsolved problems as to the taxonomy of the genus *Erwinia*.¹⁹⁾ There is a distinct difference between our isolates and the known *Erwinia* species in the G + C content, i.e., 46.3 to 48.7 mol% for our isolates, as shown in Table I, and 50 to 57 mol% for the known *Erwinia* species.¹⁸⁾ These three

strains have been deposited at the ATCC under the number given in Table I.

Reaction products in the cell extracts

D-Glucose, D-gluconate, 2-keto-D-gluconate and 5-keto-D-gluconate were used as substrates. After 30-min incubation at 28°C, the products formed were determined. Despite the addition or non-addition of PMS plus DCPIP as electron acceptors, the cell extracts (0.5 mg of protein) prepared from all the above three representative strains, SHS 2003, SHS 2006 and SHS 2008, produced D-gluconate from D-glucose, 2-keto-D-gluconate from D-gluconate, and 25DKG from 2-keto-D-gluconate. The addition of PMS plus DCPIP stimulated the formation of each product. Table II summarized the results. The formation of D-gluconate, 2-keto-D-gluconate and 25DKG from D-glucose was observed on increasing the amount of protein in the reaction mixture. No formation of 5-keto-D-gluconate from D-gluconate or 25DKG from 5-keto-D-gluconate was observed. When NAD⁺ or NADP⁺ was used instead of PMS plus DCPIP, 2-keto-D-

TABLE II. PRODUCTS FORMED FROM D-GLUCOSE AND RELATED SUBSTANCES IN CELL EXTRACTS OF THE REPRESENTATIVE STRAINS, SHS 2003, SHS 2006 AND SHS 2008, IDENTIFIED BY PAPER AND GAS CHROMATOGRAPHY

Strain	Substrate ^a	Electron acceptors ^b	Products detected ^{a,c}			
			DGA	2KDG	5KDG	25DKG
SHS 2003	GLU	PMS	+	—	—	—
2006		plus	+	—	—	—
2008		DCPIP	+	—	—	—
SHS 2003	DGA	PMS		+	—	—
2006		plus		+	—	—
2008		DCPIP		+	—	—
SHS 2003	2KDG	PMS	—		—	+
2006		plus	—		—	+
2008		DCPIP	—		—	+
SHS 2003	5KDG	PMS	—	—		—
2006		plus	—	—		—
2008		DCPIP	—	—		—

^a GLU, D-glucose; DGA, D-gluconate; 2KDG, 2-keto-D-gluconate; 5KDG, 5-keto-D-gluconate.

^b PMS, phenazine methosulfate; DCPIP, 2,6-dichlorophenol indophenol.

^c +, detected; —, not detected.

gluconate was produced slightly from D-gluconate in the soluble fractions of the cell extracts of all three strains in the presence of 1.0 mM KCN.

Dehydrogenating activities in the soluble and particulate fractions

To clarify the location of the enzyme(s) responsible for the oxidation of D-glucose to 25DKG, the soluble and particulate fractions of the cell extracts prepared from the above three representative strains, SHS 2003, SHS 2006 and SHS 2008, were further examined. Table III shows the results obtained when PMS plus DCPIP was used as electron acceptors. More than 90% of the D-glucose dehydrogenating activities, more than 82% of the D-gluconate dehydrogenating activities and more than 80% of the 2-keto-D-gluconate dehydrogenating activities of the cell extracts of the three strains were found in the respective particulate fractions.

TABLE III. DISTRIBUTION OF ACTIVITIES OF D-GLUCOSE DEHYDROGENATION (GLDH), D-GLUCONATE DEHYDROGENATION, (GADH) AND 2-KETO-D-GLUCONATE DEHYDROGENATION (2KDDH) IN CELL EXTRACTS OF THE REPRESENTATIVE STRAINS, SHS 2003, SHS 2006 AND SHS 2008^{a, b}

Strain	Fraction	Distribution of activities (%)		
		GLDH	GADH	2KDDH
SHS 2003	Soluble	2.5	8.0	17.8
	Particulate	97.5	92.0	82.2
SHS 2006	Soluble	3.4	15.0	6.8
	Particulate	96.6	85.0	93.2
SHS 2008	Soluble	9.9	17.6	19.4
	Particulate	90.1	82.4	80.6

^a Total activities of GLDH, GADH and 2KDDH in the cell extracts (Units/ml): 37.76, 47.82 and 21.08 for strain 2003; 17.60, 14.48 and 29.38 for strain 2006; and 9.04, 20.86 and 22.56 for strain 2008. One unit was defined as the amount of enzyme reducing 1 μ mol of 2,6-dichlorophenolindophenol per minute at 28°C.

^b The concentrations of protein in the cell extracts (mg/ml); 15.80 for strain 2003, 17.22 for strain 2006 and 11.06 for strain 2008.

DISCUSSION

Ninety-six bacterial strains that produced more or less Ca-25DKG from 25% (w/v) D-glucose in baffled flasks were isolated from 235 soil, fruit and vegetable samples. Using a few strains chosen from among them, we studied a number of fermentation parameters in order to optimize the Ca-25DKG production. The parameters included inoculum size, aeration, timing of transfer and timing of D-glucose feeding. The most effective results as to Ca-25DKG production were obtained by feeding D-glucose as described previously.¹⁾ Under these conditions, 42 strains among the 96 strains produced Ca-25DKG from 30% (w/v) D-glucose with excellent yields of more than 70 mol% in 1-l fermentors containing a simple medium, e.g., D-glucose, corn steep liquor, KH₂PO₄, MgSO₄·7H₂O, CaCO₃ and tap water. The fermentations were all completed within 40 hr.

Interestingly, all of the 42 strains are small rod-shaped, gram-negative, non-spore-forming and facultatively anaerobic bacteria, and can be classified taxonomically into three groups. Representative strains, SHS 2003, SHS 2006 and SHS 2008, were identified as members of the genus *Erwinia* as described under RESULTS. The ability of microorganisms to oxidize D-glucose to 25DKG has been reported for gram-negative and strictly aerobic bacteria belonging to the genera *Acetobacter*,^{2~4)} *Gluconobacter*⁵⁾ and *Acetomonas*.⁶⁾ Since our isolates were able to grow under anaerobic conditions but could not grow at pH 4.5, they could not be assigned as members of the genus *Acetobacter* or *Gluconobacter*. In addition, the G+C contents of our representative strains (46.3 to 48.7 mol%) were lower than those of the above two genera (51 to 64 mol%).^{25, 26)} Recently, *Acetomonas albosamesae* (Wakisaka) ATCC No. 21998⁶⁾ has been listed as *Erwinia herbicola* in the ATCC catalogue.¹⁴⁾ Its G+C content (55.5 mol%), however, is higher than those of our isolates. Bernaerts and De Ley²⁷⁾ also reported that a few strains of *Chromobacte-*

rium (gram-negative and facultatively anaerobic) produced small amounts of 25DKG from D-gluconate. Our isolates, however, differ in oxidase activity and G+C content, which are positive and 50 to 68 mol%, respectively, for *Chromobacterium*.²⁸⁾ From these differences, it seems that bacteria capable of producing 25DKG in high concentrations with high yields from D-glucose are usually small rod-shaped, gram-negative, non-spore-forming and facultatively anaerobic bacteria, *Erwinia* species, with G+C contents, 46.3 to 48.7 mol%, lower than those of the known *Erwinia* species (50 to 57 mol%).¹⁸⁾

The oxidation of D-glucose to 25DKG via D-gluconate and 2-keto-D-gluconate has been reported for *A. melanogenum* MA 6.2,³⁾ *P. albosamae*⁷⁾ and pigment-producing strains of *Gluconobacter*.⁵⁾ Another pathway of D-glucose to 25DKG via D-gluconate and 5-keto-D-gluconate has been proposed for *A. melanogenus* ATCC 9937.⁴⁾ All our isolates accumulated D-gluconate and 2-keto-D-gluconate as intermediate products from D-glucose, whereas no 5 keto-D-gluconate could be detected in the culture broth throughout their cultivation. To clarify the metabolic pathway for D-glucose, the reaction products with the cell extracts of the three representative strains were analyzed in the presence of electron acceptors. D-Gluconate was produced from D-glucose, 2-keto-D-gluconate from D-gluconate and 25DKG from 2-keto-D-gluconate. However, 5-keto-D-gluconate was not produced from D-gluconate, nor 25DKG from 5-keto-D-gluconate. These results suggested that our isolates oxidize D-glucose to 25DKG via D-gluconate and 2-keto-D-gluconate, i.e., via a pathway similar to that in *A. melanogenum* MA 6.2, *P. albosamae* and pigment-producing strains of *Gluconobacter*.

In pigment-producing strains of *Gluconobacter*,⁵⁾ 25DKG production has been reported to be catalyzed by membrane-bound enzymes (particulate enzymes). In our three representative strains, most activities of enzyme(s) responsible for the dehydrogenation

of D-glucose, D-gluconate and 2-keto-D-gluconate were also found in the particulate fractions, thus indicating that the enzyme(s) are membrane-bound.

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