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Efficient Production of 2,5-Diketo-D-Gluconate via Heterologous Expression of 2-Ketogluconate Dehydrogenase in *Gluconobacter japonicus*

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2,5-Diketo-D-gluconate (2,5DKG) is a compound that can be the intermediate for D-tartrate and also vitamin C production. Although *Gluconobacter oxydans* NBRC3293 produces 2,5DKG from D-glucose via D-gluconate and 2-keto-D-gluconate (2KG), with accumulation of the product in the culture medium, the efficiency of 2,5DKG production is unsatisfactory because there is a large amount of residual D-gluconate at the end of the biotransformation process. Oxidation of 2KG to 2,5DKG is catalyzed by a membrane-bound flavoprotein-cytochrome *c* complex: 2-keto-gluconate dehydrogenase (2KGDH). Here, we studied the *kgdSLC* genes encoding 2KGDH in *G. oxydans* NBRC3293 to improve 2,5DKG production by *Gluconobacter* spp. The *kgdS*, *kgdL*, and *kgdC* genes correspond to the small, large, and cytochrome subunits of 2KGDH, respectively. The *kgdSLC* genes were cloned into a broad-host-range vector carrying a DNA fragment of the putative promoter region of the membrane-bound alcohol dehydrogenase gene of *G. oxydans* for expression in *Gluconobacter* spp. According to our results, 2KGDH that was purified from the recombinant *Gluconobacter* cells showed characteristics nearly the same as those reported previously. We also expressed the *kgdSLC* genes in a mutant strain of *Gluconobacter japonicus* NBRC3271 (formerly *Gluconobacter dioxyacetonicus* IFO3271) engineered to produce 2KG efficiently from a mixture of D-glucose and D-gluconate. This mutant strain consumed almost all of the starting materials (D-glucose and D-gluconate) to produce 2,5DKG quantitatively as a seemingly unique metabolite. To our knowledge, this is the first report of a *Gluconobacter* strain that produces 2,5DKG efficiently and homogeneously.

Gluconobacter strains are Gram-negative obligate aerobes belonging to the group of acetic acid bacteria. They have unique characteristics that help them to carry out incomplete oxidation of a wide variety of carbohydrates (using membrane-bound dehydrogenases), leading to the accumulation of large amounts of the corresponding oxidized compounds in the culture medium (1). Membrane-bound dehydrogenases are located in the cytoplasmic membrane facing the periplasm and are linked to the respiratory chain. In this system, the electrons accepted from the substrates are transferred to ubiquinone and then to terminal ubiquinol oxidases to generate the proton motive force, which serves as biological energy for microorganisms (1).

2-Keto-D-gluconate (2KG) and 5-keto-D-gluconate (5KG) production from D-glucose in *Gluconobacter* strains is catalyzed by a series of membrane-bound enzymes. Membrane-bound glucose dehydrogenase (mGDH; encoded by the gene *gdhM*) is a quinoprotein that contains pyrroloquinoline quinone (PQQ) as a prosthetic group and oxidizes D-glucose to D-glucono-δ-lactone, which is subsequently converted to D-gluconate spontaneously or by gluconolactonase (2, 3). D-Gluconate is oxidized to 5KG and 2KG by PQQ-dependent sorbitol dehydrogenase (also referred to as PQQ-dependent glycerol dehydrogenase [PQQ-GLDH]) and gluconate 2-dehydrogenase (GADH), respectively.

PQQ-GLDH was purified by at least two independent research teams. First, our group reported the isolation of PQQ-GLDH as a glycerol dehydrogenase from *Gluconobacter japonicus* NBRC3260 (formerly *Gluconobacter industrius* IFO3260) (4). Second, Sugisawa and Hoshino reported the isolation of PQQ-GLDH as a sorbitol dehydrogenase from *Gluconobacter thailandicus* NBRC3255 (formerly *Gluconobacter suboxydans* IFO3255) (5). The *sldBA* genes encoding PQQ-GLDH were cloned by Hoshino's group (6). This enzyme oxidizes a broad range of substrates, including D-gluconate, glycerol, and D-sorbitol, with high regio- and stereospecificity and thereby produces the corresponding ketones (7).

GADH of *Gluconobacter japonicus* NBRC3271 (formerly *Gluconobacter dioxyacetonicus* NBRC3271) is a flavoprotein-cytochrome *c* complex containing flavin adenine dinucleotide (FAD) and heme C as prosthetic groups (8). The substrate specificity of purified GADH, with a Michaelis constant of 2.2 mM, is strictly limited to D-gluconate. Recently, it was reported that two types of GADHs that are encoded by *gndSLC* and *gndFGH* function in *Gluconobacter* strains (9, 10).

Some *Gluconobacter* and *Gluconacetobacter* strains oxidize 2KG further to 2,5-diketo-D-gluconate (2,5DKG) by means of 2-keto-gluconate dehydrogenase (2KGDH) (Fig. 1) (11, 12). This enzyme was purified from *Gluconobacter oxydans* NBRC3293

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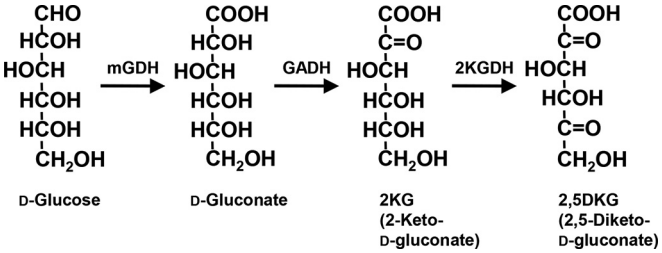

FIG 1 Pathway of 2,5DKG production from D-glucose in *Gluconobacter*. mGDH, membrane-bound glucose dehydrogenase; GADH, gluconate 2-dehydrogenase; 2KGDH, 2-keto-gluconate dehydrogenase.

(formerly *Gluconobacter melanogenus* IFO3293) and characterized as a flavoprotein-cytochrome *c* complex (molecular mass, ~133 kDa) consisting of three subunits: a large subunit (61 kDa) with covalently bound FAD, a cytochrome subunit (47 kDa) with covalently bound heme C, and a small subunit (25 kDa) (13, 14). Purified 2KGDH shows strict substrate specificity for 2KG with a Michaelis constant of 50 mM for the production of 2,5DKG.

2,5DKG is not only the precursor for D-tartrate production but also potentially the intermediate for L-ascorbate (vitamin C) production because 2,5DKG can be converted to 2-keto-L-gulonate (2KLG), a precursor of vitamin C, via a single reduction reaction (15–18). Therefore, several attempts were made to produce 2,5DKG in various microorganisms (19, 20). However, current industrial vitamin C production is done by using D-sorbitol as the feedstock via two-step fermentation and single chemical processes; first, D-sorbitol is regiospecifically oxidized to L-sorbose by *G. oxydans*; second, L-sorbose is converted to 2KLG by cocultivation of *Ketogulonicigenium vulgare* with *Bacillus megaterium* as a supplier of growth factor; and finally, 2KLG is chemically converted to vitamin C (21). In this process, the titer of 2KLG reaches 515 mM (100 g · liter^{-1}) and the overall yield of 2KLG from D-sorbitol is reported to be approximately 90% (21). On the other hand, one of the promising methods for 2,5DKG production is the oxidation of D-glucose by *Erwinia* sp., where D-glucose can be converted to 2,5DKG with a titer and yield of 1.59 M (328.6 g · liter^{-1} Ca-2,5DKG) and 94.5%, respectively, during a 26-h fermentation (19). Even though the *Erwinia citreus* strain that overexpresses the gene encoding 2,5DKG reductase from *Corynebacterium* sp. directly produces 2KLG from D-glucose, the titer and yield are 102 mM (19.8 g · liter^{-1}) and 49.4%, respectively (17). Such low productivity is likely uncompetitive with the method using *K. vulgare*.

In the present study, we found that *G. oxydans* NBRC3293 produces 2,5DKG from a mixture of D-glucose and D-gluconate inefficiently because a significant amount of residual D-gluconate remains in the culture medium and 5KG is also produced. Because we have developed a *G. japonicus* strain producing 2KG efficiently, we anticipate that heterologous expression of the genes of 2KGDH in the recombinant strain of *G. japonicus* will result in a *Gluconobacter* strain that produces 2,5DKG with a high efficiency. Hence, we decided to identify the genes of 2KGDH in *G. oxydans* strain NBRC3293.

MATERIALS AND METHODS

Materials. 2KG hemicalcium salt was purchased from Sigma-Aldrich (St. Louis, MO, USA). Masaaki Tazoe and Tatsuo Hoshino (NRL Pharma, Inc., Kawasaki, Japan) generously provided us with 2,5DKG hemicalcium salt as a gift. The restriction endonucleases and modification enzymes for genetic engineering either were gifts from Toyobo (Osaka, Japan) or were purchased from Agilent Technologies (Santa Clara, CA, USA). Yeast extract was a generous gift from Oriental Yeast (Osaka, Japan). All other materials were obtained from commercial sources.

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. We used *G. oxydans* strain NBRC3293, the *G. oxydans* NBRC12528 Δ*adhA*::Km^r derivative (22), and the *G. japonicus* NBRC3271 Δ*sldA*::Km^r derivative (9) in this study. The broad-host-range vector pSHO8 (23) was used for the expression of the *kgdSLC* genes of *G. oxydans* NBRC3293 in *G. oxydans* NBRC12528 Δ*adhA*::Km^r and *G. japonicus* NBRC3271 Δ*sldA*::Km^r. *Gluconobacter* strains were grown as a preculture in ΔP medium (5 g of D-glucose, 20 g of glycerol, 10 g of polypeptone, 10 g of yeast extract per liter) at 30°C for 24 h with vigorous shaking. Kanamycin and ampicillin were used at final concentrations of 50 μg · ml^{-1} and 250 μg · ml^{-1}, respectively.

Escherichia coli strain DH5α was used for plasmid construction. *E. coli* HB101 harboring pRK2013 was used as a helper strain for conjugative plasmid transfer, which was performed using a triparental mating method. *E. coli* strains were grown in modified Luria-Bertani medium (10

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Escherichia coli		
DH5α	F$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*ΔM15 λ$^-$	43
HB101	F$^-$ *thi-1 hsdS20* (r_B^- m_B^-) *supE44 recA13 ara14 leuB6 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1* λ$^-$	44
Gluconobacter oxydans		
NBRC3293	Wild type	NBRC[a]
NBRC12528 Δ*adhA*	NBRC12528 Δ*adhA*::Km^r	22
Gluconobacter japonicus NBRC3271 Δ*sldA*	NBRC3271 Δ*sldA*::Km^r	9
Plasmids		
pKR2013	Plasmid mediates conjugation-based plasmid transfer; Km^r	45
pSHO8	Broad-host-range plasmid with a putative promoter region of the *adhAB* gene of *G. oxydans* ATCC 621H; *mob* Ap^r	23
pNK316	pSHO8, a 3.8-kb fragment of the *kgdSLC* genes of *G. oxydans* NBRC3293	This study

[a] NBRC, National Biological Resource Center, National Institute of Technology and Evaluation (Japan; http://www.nbrc.nite.go.jp/).

g of polypeptone, 5 g of yeast extract, 5 g of NaCl per 1 liter in distilled water), and the pH was adjusted to 7.0 with NaOH. Ampicillin was used at a final concentration of 50 $\mu g \cdot ml^{-1}$.

Construction of the plasmid. The *kgdSLC* genes of *G. oxydans* NBRC3293 were amplified by means of Herculase II fusion DNA polymerase (Agilent Technologies) using a genomic DNA sample from *G. oxydans* NBRC3293 and the primers 316-F (5′-CTCGAGGAAATGACA ATGATCTCTGT-3′; the XhoI site is underlined) and 316-R (5′-AGATC TTCAGGCCTTCGCCTTTCGGG-3′; the BglII site is underlined). To construct the pNK316 plasmid, the PCR products digested with BglII and XhoI were inserted by ligation into the pSHO8 vector (23) that had been digested with BamHI and SalI.

Preparation of the cell suspension and membrane fraction. The bacterial cells were cultivated in a 2,5DKG production medium consisting of 10 g of D-glucose, 10 g of Na-D-gluconate, 3 g of polypeptone, and 3 g of yeast extract per liter. After precultivation under the conditions described above, the preculture was added at 1.0% (vol/vol) into 100 ml of the medium in a 500-ml flask, and the cultivation was carried out at 30°C with vigorous shaking for 24 h. After centrifugation to collect the cells, the cells were washed with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH buffer (pH 6.0) containing 1 mM $CaCl_2$, which may prevent the release of PQQ from mGDH. The washed cells were resuspended in appropriate volumes of the buffer, and the resulting cell suspension was used for the oxidase assays.

The medium for membrane preparation consisted of 5 g of Na-D-gluconate, 5 g of D-glucose, 3 g of polypeptone, and 3 g of yeast extract per liter (13). After precultivation, the preculture was added at 1.0% (vol/vol) into 100 ml of the medium in a 500-ml flask, and the cultivation was carried out at 30°C for 24 h with vigorous shaking. The cells in the cultured broth were collected by centrifugation at 10,000 × *g* and 4°C for 10 min and were washed with 10 mM potassium phosphate buffer (pH 6.0). The washed cells were resuspended in appropriate volumes of the buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cells in the suspension were disrupted by 2 passages through a French pressure cell press. After the cell debris was precipitated by centrifugation at 10,000 × *g* and 4°C for 10 min, the supernatant was ultracentrifuged at 100,000 × *g* and 4°C for 1 h. The precipitate was resuspended in appropriate volumes of the buffer containing 0.5 mM PMSF and used as the membrane fraction in the experiments described below.

Enzymatic assays. All enzymatic reactions were carried out at 25°C. Appropriate buffers and substrate concentrations were selected for different substrates: 25 mM 2KG in McIlvaine buffer (a mixture of 0.1 M citric acid and 0.2 M disodium hydrogen phosphate, pH 4.0), 100 mM D-gluconate in McIlvaine buffer (pH 5.0), and 100 mM D-glucose in 45 mM potassium phosphate buffer (pH 6.0). Dehydrogenase activity was measured using potassium ferricyanide as the electron acceptor as described previously (24). The activity of 2,3-dimethoxy-5-farnesyl-1,4-benzoquinone (Q-1) reductase was measured by monitoring the decrease in absorbance at 275 nm in a reaction mixture consisting of appropriate amounts of purified 2KGDH, 25 mM 2KG, 7.9 mM NaN_3, and 30 μM Q-1 dissolved in dimethyl sulfoxide (DMSO; final DMSO concentration, 0.2% [vol/vol]) in McIlvaine buffer (pH 4.0) (25). One unit (U) of enzyme activity was defined as the amount of the enzyme oxidizing 1 μmol of the substrate per min. Oxidase activity was measured by oxygen uptake using a Clark-type oxygen electrode. One unit of oxidase activity was defined as the amount of the enzyme consuming 0.5 μmol of molecular oxygen per min, assuming that O_2 is dissolved in the assay buffer at 498 $\mu mol \cdot liter^{-1}$. The membrane fractions and cell suspensions were used for the dehydrogenase and oxidase assays, respectively. The protein concentration was measured by means of the modified Lowry method (26). Bovine serum albumin was used as the standard protein.

Purification of 2KGDH. Solubilization and purification of 2KGDH were performed as described previously (13) with some modifications, as follows. The membranes were resuspended in 10 mM potassium phosphate buffer (pH 6.0) at a concentration of 10 mg of protein per ml with addition of 300 mM KCl and 2.0% (wt/vol) Triton X-100, and the mixture was gently stirred at 4°C for 1 h. We then isolated 2KGDH from the supernatant fraction resulting from ultracentrifugation at 100,000 × *g* and 4°C for 1 h. The supernatant fraction was applied to a hydroxyapatite column equilibrated with 10 mM potassium phosphate buffer (pH 6.0) containing 0.1% (wt/vol) Triton X-100. The elution of 2KGDH was carried out using a concentration gradient of potassium phosphate buffer (pH 6.0) from 10 mM to 100 mM containing 0.1% (wt/vol) Triton X-100, and the active fractions were collected. The enzyme solution was dialyzed against 10 mM sodium acetate buffer (pH 5.5; containing 0.1% [wt/vol] Triton X-100) at 4°C for 12 h. After centrifugation at 10,000 × *g* and 4°C for 10 min, the supernatant was applied to a CM-Toyopearl column equilibrated with 10 mM sodium acetate buffer (pH 5.5) containing 0.1% (wt/vol) Triton X-100. Next, 2KGDH was eluted with a linear gradient of NaCl (in the same buffer) from 0 to 200 mM. The purity was tested using SDS-PAGE, and the gel was stained with Coomassie brilliant blue R-250. The fraction that contained the fewest impurities was used as purified 2KGDH.

Determination of the N-terminal amino acid sequence of the small subunit. Proteins in the gel were transferred electrophoretically onto a polyvinylidene difluoride membrane and stained with Coomassie brilliant blue R-250. The protein band corresponding to the small subunit was cut off. The N-terminal amino acid sequence was analyzed with a Procise 494 HT peptide sequencer (Life Technologies, Carlsbad, CA).

2,5DKG production. The cells were precultivated in a 2,5DKG production medium consisting of 10 g of D-glucose, 10 g of Na-D-gluconate, 3 g of polypeptone, and 3 g of yeast extract per liter. One milliliter of the preculture was transferred into 100 ml of the medium in a 500-ml flask, and the cultivation was carried out at 30°C with vigorous shaking.

Analytical procedures. Bacterial growth was measured using a spectrophotometer. All metabolites were analyzed by means of a high-performance liquid chromatography (HPLC) system equipped with a refraction index (RI) detector. D-Glucose was quantified on a Pb^{2+}-loaded cation-exchange column (8.0 mm [inside diameter {i.d.}] by 300 mm [length]; Sugar SP0810 Shodex; Showa Denko KK, Kawasaki, Japan) at 80°C using distilled and deionized water as the mobile phase at a flow rate of 0.5 $ml \cdot min^{-1}$. The retention time of D-glucose was 20.3 min. D-Gluconate, 5KG, 2KG, and 2,5DKG were quantified on an ion-exclusion column (RSpak KC-811, 8.0 mm [i.d.] by 300 mm [length]; Shodex, Showa Denko KK, Kawasaki, Japan) at 60°C using 0.1% (wt/vol) phosphoric acid as the mobile phase at a flow rate of 0.7 $ml \cdot min^{-1}$. The retention times of D-gluconate, 5KG, 2KG, and 2,5DKG were 11.3, 10.9, 10.5, and 10.1 min, respectively. Because the retention time of D-glucose was 11.3 min on the ion-exclusion column, the concentrations of D-gluconate were calculated by subtracting the D-glucose concentrations determined by use of the Pb^{2+}-loaded cation-exchange column from the concentrations determined by use of the ion-exclusion column. Calibration curves were constructed by means of peak heights using different concentrations of the compounds ranging from 0 to 100 mM D-glucose and D-gluconate, from 0 to 50 mM 5KG and 2KG, and from 0 to 45 mM 2,5DKG.

Heme staining was conducted to visualize proteins that covalently bind heme C moieties in the SDS-polyacrylamide gel without reducing agent by heme-catalyzed peroxidase activity (27). Covalently bound FAD was examined by exposing the SDS-polyacrylamide gel without reducing agent to UV light (312 nm; TX-15MP; Atto, Tokyo, Japan).

Bioinformatic analyses. For phylogenetic analysis, sequences were aligned at the amino acid level using MUSCLE, version 3.8.31, software (28, 29). Poorly aligned regions were removed using GBLOCKS, version 0.91b, software (30–32). The RAxML, version 8.0.14, software package was used for maximum likelihood analyses, using both the fast and the standard bootstrap algorithms with 500 replicates (32). The PROTGAMMAWAG model was implemented for optimization of individual per site substitution rates. The tree was drawn graphically in the MEGA, version 5.05, program (33, 34). Genetyx-Mac software (version 17; Genetyx, Tokyo, Japan) was used for cal-

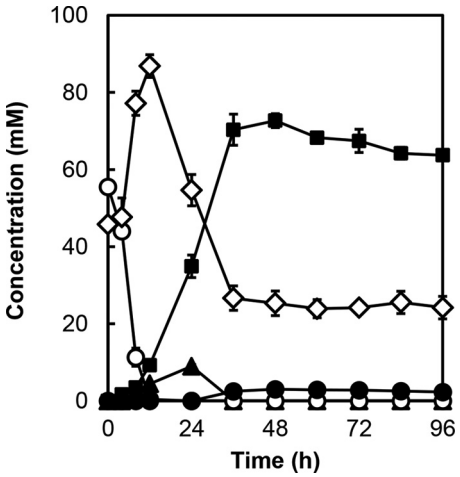

FIG 2 Consumption of D-glucose and D-gluconate and formation of 5KG, 2KG, and 2,5DKG by *Gluconobacter oxydans* NBRC3293. The cells were precultivated in 2.5 ml of ΔP medium at 30°C for 24 h and then transferred to 100 ml of the 2,5DKG production medium. Mean values and standard deviations from triplicate experiments are shown. Symbols: open circles, D-glucose; open diamonds, D-gluconate; filled circles, 5KG; filled triangles, 2KG; filled squares, 2,5DKG.

culations of global identity among predicted amino acid sequences and predicted molecular masses and for prediction of signal peptide cleavage sites.

Nucleotide sequence accession number. The nucleotide sequence of the *kgdSLC* genes of *G. oxydans* strain NBRC3293 and its predicted amino acid sequence were deposited in DDBJ/EMBL/GenBank under accession number AB985494.

RESULTS

Oxidation of D-gluconate is a rate-limiting step in 2,5DKG production by wild-type *G. oxydans* NBRC3293. In order to assess the 2,5DKG production efficiency of *G. oxydans* NBRC3293, a biotransformation experiment was carried out in the 2,5DKG production medium containing D-glucose and D-gluconate to monitor growth, pH, and metabolites. *G. oxydans* NBRC3293 grew, and the culture reached an absorbance at 600 nm (A_{600}) of 3.98 ± 0.13 (mean ± standard deviation [SD]) at 96 h; the pH dropped during the exponential phase of growth, and the final pH value of the medium was 3.5 ± 0.0 (see Fig. S1 in the supplemental material).

The time course of formation of the products of *G. oxydans* NBRC3293 in the 2,5DKG production medium is shown in Fig. 2. *G. oxydans* NBRC3293 consumed D-glucose within the first 12 h, resulting in an increase in the D-gluconate concentration and the formation of 2KG and 5KG. The 2,5DKG was produced as expected, and the maximum concentration of 72.7 ± 1.8 mM was obtained at 48 h. Nonetheless, the amount of 2,5DKG accumulated in the medium gradually decreased to 63.7 ± 1.1 mM at 96 h. 5KG was also produced as a by-product, and the final concentration was 2.27 ± 0.050 mM.

No D-gluconate consumption was detected after 36 h, with 24.2 ± 2.9 mM D-gluconate (approximately one-quarter of the original D-glucose and D-gluconate present) remaining at 96 h. Thus, 2,5DKG production by *G. oxydans* NBRC3293 was heterogeneous; the products contained substantial amounts of D-gluconates and 5KG as minor by-products in our biotransformation experiment. We assumed that the incomplete consumption of D-gluconate would prevent the efficient production of 2,5DKG by *G. oxydans* NBRC3293 and the downstream process for purification of 2,5DKG.

In order to achieve a process of efficient and homogeneous production of 2,5DKG, we decided to create a bacterial strain with a strong ability to convert D-glucose to 2,5DKG but with a lack of ability to produce 5KG. In our previous study, we constructed *G. japonicus* strain NBRC3271 Δ*sldA*, which lacks PQQ-GLDH, for catalyzing the oxidation of D-gluconate to 5KG and, consequently, efficiently produces 2KG as a unique metabolite (9). The GADH activity of the Δ*sldA* strain was 2.35 ± 0.040 U · (mg membrane protein)$^{-1}$, which was 10-fold higher than that of *G. oxydans* NBRC3293 [0.240 ± 0.010 U · (mg membrane protein)$^{-1}$; Table 2]. Therefore, our strategy for strain construction was heterologous expression of the genes encoding 2KGDH in *G. japonicus* NBRC3271 Δ*sldA*. For this purpose, we first characterized the genes encoding 2KGDH in *G. oxydans* NBRC3293.

Identification of genes encoding 2KGDH in *G. oxydans* NBRC3293. 2KGDH of *G. oxydans* NBRC3293 was purified and characterized as a flavoprotein-cytochrome *c* complex consisting of three subunits—large, small, and cytochrome subunits (13)—but the genes of this enzyme in acetic acid bacteria have not been identified yet. On the other hand, Pujol and Kado (35) proposed candidate structural genes of 2KGDH in *Pantoea citrea* by means of genetic analysis and transposon mutagenesis. The gene cluster in question encodes three polypeptides corresponding to the small, large, and cytochrome subunits, just as in other heterotrimeric flavoprotein-cytochrome *c* complexes, such as D-gluconate dehydrogenase. Because we obtained the draft genome sequence of *G. oxydans* NBRC3293 (M. Matsutani and K. Matsushita, unpublished data), we used the genome information to identify the genes encoding 2KGDH. We found a gene (named *kgdL*) homologous to the gene of the large subunit of *P. citrea* 2KGDH as a candidate gene of the large subunit of *G. oxydans* 2KGDH by means of a homology search using the Basic Local Alignment Search Tool (BLAST) program (36). The global identity of the amino acid sequence of KgdL to that of the large subunit of *P. citrea* 2KGDH was 74%. The *kgdL* gene likely constitutes a gene cluster with the genes named *kgdS* and *kgdC*, i.e., the *kgdSLC* gene cluster. KgdS and KgdC showed global identities of 25% and 52% to the precursor forms of the small and cytochrome subunits of *P. citrea* 2KGDH, respectively.

In order to examine their functions, we cloned the *kgdSLC* genes and expressed them in *G. oxydans* NBRC12528, which does not have 2KGDH activity. In addition, because ADH is a major membrane protein in NBRC12528 and is likely to interfere with 2KGDH purification, we used a derivative strain lacking ADH (Δ*adhA*::Km^r) in this study. A broad-host-range plasmid carrying a putative promoter region for *adhAB* (*gox1067* and *gox1068*) of *G. oxydans* 621H was used for expression of the *kgdSLC* genes (23).

TABLE 2 Dehydrogenase activity in membrane fractions of *G. oxydans* strain NBRC3293 and *G. japonicus* strain NBRC3271 Δ*sldA*

Strain	Mean sp act ± SD [U · (mg membrane protein)$^{-1}$]	
	D-Gluconate	2-Keto-D-gluconate
G. oxydans NBRC3293	0.240 ± 0.010	0.720 ± 0.020
G. japonicus NBRC3271 Δ*sldA*	2.35 ± 0.040	ND[a]

[a] ND, not detected.

G. oxydans NBRC3293 showed 2KGDH activity of 0.720 ± 0.020 U · (mg membrane protein)$^{-1}$ in the membrane fractions (Table 2). The membrane fractions of the NBRC12528 Δ*adhA* cells harboring pNK316 (*kgdSLC*) showed 2KGDH activity of 37.7 ± 1.4 U · (mg membrane protein)$^{-1}$. This value was approximately 50-fold higher than that for *G. oxydans* NBRC3293. The membrane fractions of the NBRC12528 Δ*adhA* strain harboring pSHO8 did not show any 2KGDH activity. The SDS-polyacrylamide gel stained by means of heme-catalyzed peroxidase showed an intense band of approximately 48 kDa in the membrane fractions of the Δ*adhA* strain harboring pNK316 but no band in those of the reference strain (data not shown). Taken together, these results suggest that *kgdSLC* represent the genes of the small, large, and cytochrome subunits of 2KGDH in *G. oxydans* NBRC3293.

Properties of the *kgdSLC* genes and the encoded proteins. The nucleotide sequence of the *kgdSLC* gene cluster and its predicted amino acid sequence are shown in Fig. S2 in the supplemental material. Each candidate Shine-Dalgarno sequence was identified upstream of the putative initiation codon. The small-subunit protein KgdS has the amino acid sequence GRRSLLL in the N-terminal region, a possible signal sequence for the twin-arginine translocation (Tat) system that translocates secretory proteins across the cytoplasmic membrane (37). In contrast, the large-subunit protein KgdL has no signal sequence; this finding is suggestive of a hitchhiker mechanism involving the small subunit for translocation. The *kgdS* gene encoded 222 amino acids, but there were 176 amino acids for the mature protein, of which the calculated molecular mass was approximately 19 kDa (38).

The large subunit has the N-terminal sequence GSGVVGG, corresponding to the FAD binding motif GXGXXG (39). The *kgdL* gene encoded a polypeptide of 552 amino acid residues with a calculated molecular mass of approximately 60 kDa that was assembled with and covalently bound to FAD.

The cytochrome-subunit protein KgdC has a 31-amino-acid, typical Sec-dependent signal sequence at the N terminus (38, 40). The molecular mass of the proposed mature protein was calculated to be approximately 48 kDa, with the sequence being composed of 445 amino acids, but it should be higher because the deduced amino acid sequence was revealed to have three CXXCH sequence motifs for heme C-binding sites. KgdC has three heme C-binding motifs (CXXCH): $C_{22}AACH_{26}$, $C_{170}DTCH_{174}$, and $C_{307}AACH_{311}$ (the numbers correspond to those of the proposed mature form of KgdC). These properties are similar to those of all heterotrimeric membrane-bound flavoprotein dehydrogenase-cytochrome *c* complexes found in *Gluconobacter* strains, such as sorbitol dehydrogenase (SLDH), fructose dehydrogenase (FDH), and GADH (9, 10, 23, 41). By using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/), one transmembrane helix was predicted nearly in the C terminus: from the 415th to the 437th residues in the proposed mature form of KgdC. No transmembrane helices were found in two other subunits with the prediction program. This method incorporates not only hydrophobicity but also charge bias and helix length, making highly reliable predictions (42). The TMHMM prediction suggests that the cytochrome subunit anchors in the cytoplasmic membrane with its C-terminal transmembrane helix.

We examined the phylogenetic relationships among those flavoprotein dehydrogenase-cytochrome *c* complexes, including the glucose dehydrogenase of *Burkholderia cepacia* (Bc-GDH), at the subunit level (see Fig. S3 in the supplemental material). To our surprise, each subunit of Bc-GDH was deeply embedded in the phylogenetic tree (rather than being an outlier). We believe that there is a mutual lateral transfer of genes of the flavoprotein dehydrogenase-cytochrome *c* complex between *Gluconobacter* and *Burkholderia*. Albeit they were not identical, the phylogenetic patterns of all of the subunits were similar to one another, suggesting that the enzymes have developed through alterations in the genes for not only the dehydrogenase unit but also all of the subunits in the course of the evolutionary process. The phylogenetic patterns of the large and small subunits were the same, consistent with our assumption that they likely share the maturation process, in which the subcomplex of the large and small subunits is translocated to the periplasmic space through the Tat system. On the other hand, the divergence pattern of the cytochrome subunit was different from that of the other subunits; Bc-GDH diverged early from the group SLDH, 2KGDH, and FDH (see Fig. S3 in the supplemental material).

Purification and characterization of recombinant 2KGDH. We simplified the purification procedures reported previously because the concentration of 2KGDH in the membranes of NBRC12528 Δ*adhA*/pNK316 was much higher than that in NBRC3293 (13). Two fractionation steps using ammonium sulfate and polyethylene glycol 6000 were omitted from the original method of Shinagawa et al. (13).

After SDS-PAGE, the purified 2KGDH yielded three main bands of 60, 48, and 22 kDa (Fig. 3A), which are similar in size to those reported previously and correspond to the molecular masses calculated from the *kgdSLC* genes. The large subunit showed fluorescence under UV light at 312 nm, suggesting covalently bound FAD (Fig. 3B). The cytochrome subunit was stained by heme-catalyzed peroxidase activity, indicating the presence of the heme C moieties (Fig. 3C). The N-terminal amino acid sequence of the small subunit was ATPLD, as determined by Edman degradation, which corresponds to our prediction for signal sequence cleavage. The specific activity of 2KGDH purified in this study was 508 ± 2.7 U · (mg protein)$^{-1}$ at 25°C. This value is approximately half of that reported in another study where the enzyme reaction was carried out at 37°C (13). Moreover, the concentration of 2KG used for the enzymatic assay in the present study was less than that in the study just cited: 25 mM versus 100 mM.

The purified 2KGDH oxidized 2KG exclusively but was completely inert toward 5KG, D-gluconate, D-glucose, D-sorbitol, L-lactate, L-malate, and D-fructose. The enzyme showed an absorption spectrum resembling that of completely reduced cytochrome *c*; this spectrum likely comes from the heme C moieties in the cytochrome subunit. Thus, we reproduced our previous study by using the recombinant enzyme (13); this result strongly supports the notion that the *kgdSLC* genes are structural genes of 2KGDH in *G. oxydans* NBRC3293. Furthermore, the purified 2KGDH reacted with Q-1 with a specific activity of 46.9 ± 2.2 U · (mg protein)$^{-1}$, suggesting that the physiological electron acceptor of 2KGDH is ubiquinone-10 in the membrane.

Heterologous expression of *kgdSLC* in the 2KG-producing *Gluconobacter* strain enables efficient 2,5DKG production. Because the genes encoding 2KGDH of *G. oxydans* NBRC3293 were successfully identified, we tested whether expression of the *kgdSLC* genes in the 2KG producer *G. japonicus* NBRC3271 Δ*sldA* allows the efficient and homogeneous production of 2,5DKG. The recombinant strain *G. japonicus* NBRC3271 Δ*sldA*/pNK316 was cultivated in the 2,5DKG production medium, and the growth

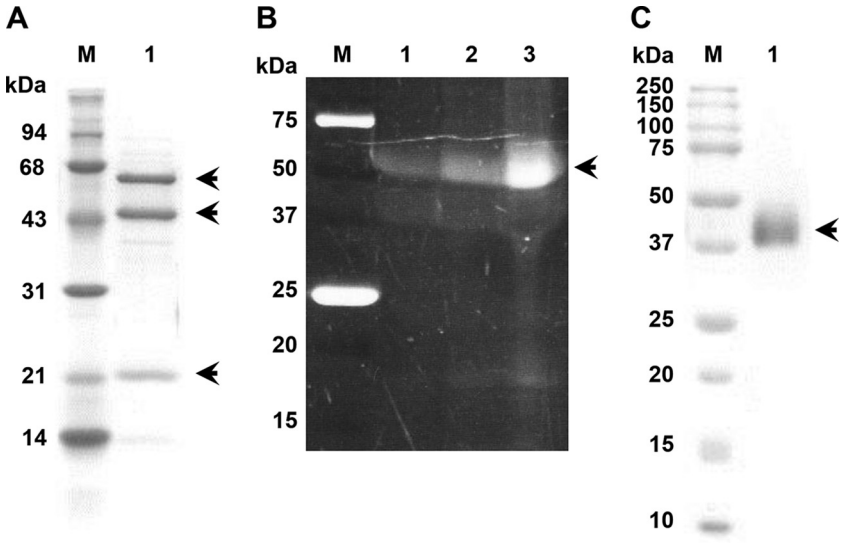

FIG 3 Properties of purified 2KGDH from *G. oxydans* NBRC12528 Δ*adhA* harboring pNK316. (A) Five micrograms of purified 2KGDH (lane 1) was subjected to SDS-PAGE, and the gel was stained with Coomassie brilliant blue R-250. Arrows, the large, cytochrome, and small subunits of 2KGDH from top to bottom, respectively. Molecular mass standards are in lane M: phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21 kDa; lysozyme, 14 kDa. (B) Fifty (lane 1), 100 (lane 2), and 200 (lane 3) micrograms of purified 2KGDH was subjected to SDS-PAGE without reducing agent, and the gel was illuminated with UV light at 312 nm. Arrow, intense fluorescence corresponding to the large subunit. (C) Five micrograms of purified 2KGDH was subjected to SDS-PAGE without reducing agent, and the gel was stained with heme-catalyzed peroxidase activity. Arrow, stained band corresponding to the cytochrome subunit. Molecular Precision Plus protein prestained standards (Bio-Rad Laboratories, Hercules, CA) were used in lanes M in panels B and C.

and pH were monitored (Fig. 4). The pH change was nearly the same for the Δ*sldA* strains harboring pSHO8 and pNK316, and the final pH of the medium was 3.2 ± 0.1. Two Δ*sldA* strains harboring pSHO8 and pNK316 showed similar growth behavior by 36 h. Nevertheless, growth of the strain harboring pNK316 increased after a 36-h cultivation, and the culture reached an A_{600} of 4.08 ± 0.16 at 96 h, whereas the strain harboring pSHO8 did not show an increase in the growth rate: it seemed to reach a plateau at 36 h, and the A_{600} at 96 h was 2.43 ± 0.12. These results show that expression of 2KGDH increased the cell yield.

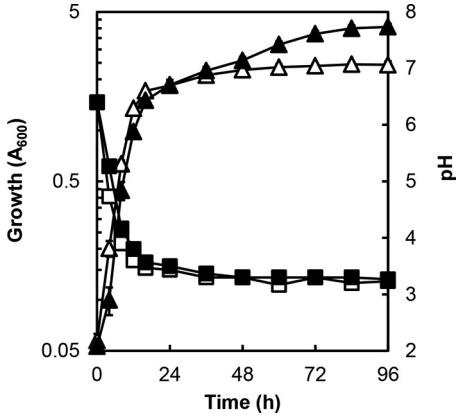

FIG 4 Growth and pH in the medium of *G. japonicus* NBRC3271 Δ*sldA* strains harboring plasmid pSHO8 (open symbols) or pNK316 (filled symbols). The cells were precultivated in 2.5 ml of ΔP medium at 30°C for 24 h and then transferred to 100 ml of the 2,5DKG production medium. The growth was monitored by measuring the A_{600}. Mean values and standard deviations from triplicate experiments are shown. Symbols: triangles, growth; squares, pH in the medium.

The oxidase activities toward D-glucose, D-gluconate, and 2KG in the Δ*sldA* strains harboring pSHO8 and pNK316 were determined in whole-cell preparations (Table 3). The Δ*sldA* strain harboring pNK316 showed a D-glucose oxidase activity of 1.00 ± 0.030 U · (mg protein)$^{-1}$ and a D-gluconate oxidase activity of 0.433 ± 0.013 U · (mg protein)$^{-1}$, which were 80.6% ± 2.4% and 89.6% ± 2.7% of those for the reference strain, respectively. A 2KG oxidase activity was detected only in the Δ*sldA* strain harboring pNK316; the specific activity was 0.179 ± 0.0090 U · (mg protein)$^{-1}$, indicating that *kgdSLC* in *G. oxydans* NBRC3293 is functionally expressed in the heterologous strain *G. japonicus* NBRC3271 Δ*sldA*, thereby constituting a 2KG-oxidizing respiratory chain.

The fermentation products of the Δ*sldA* strains harboring pSHO8 and pNK316 were determined. As shown in Fig. 5A and B, both strains consumed D-glucose within the first 12 h, resulting in an increase in the D-gluconate concentration and the formation of 2KG. D-Gluconate was consumed completely by 48 h by the strain harboring pSHO8 and by 60 h by the strain harboring pNK316. The 2KG production by the Δ*sldA* strain harboring pSHO8 reached a plateau at 48 h, and the final concentration was 87.8 ± 0.30 mM. In contrast, the strain harboring pNK316 produced

TABLE 3 Oxidase activity of *G. japonicus* NBRC3271 Δ*sldA* strains harboring plasmid pSHO8 or pNK316

Plasmid	Mean sp act ± SD [U · (mg protein)$^{-1}$]		
	D-Glucose	D-Gluconate	2-Keto-D-gluconate
pSHO8	1.24 ± 0.059	0.483 ± 0.037	ND[a]
pNK316	1.00 ± 0.030	0.433 ± 0.013	0.179 ± 0.0090

[a] ND, not detected.

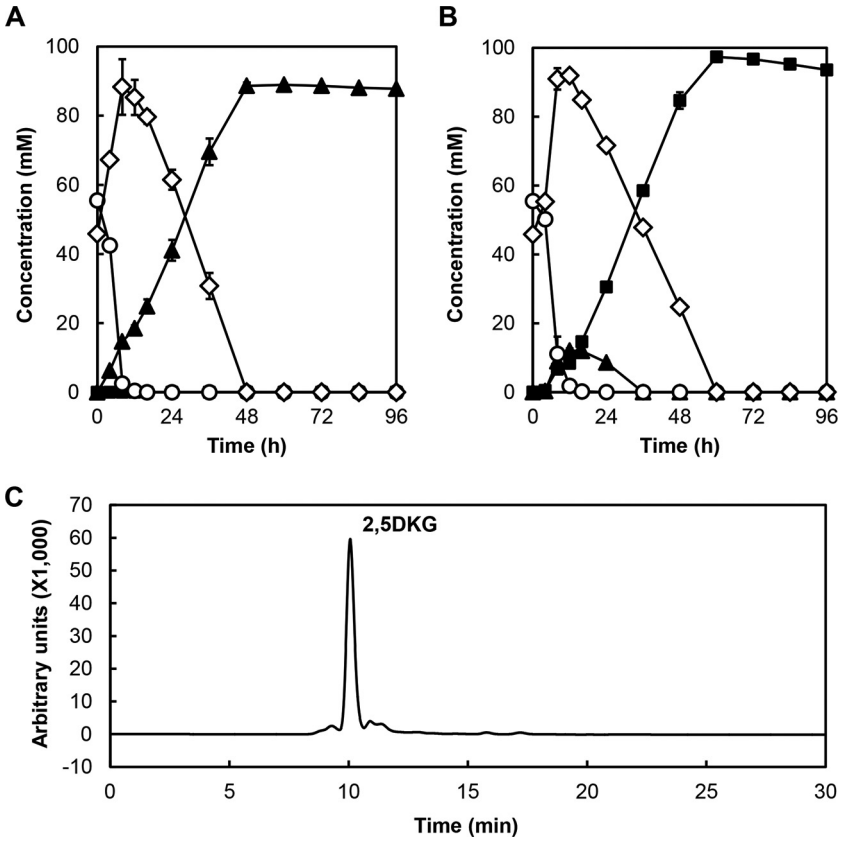

FIG 5 Consumption of D-glucose and D-gluconate and formation of 5KG, 2KG, and 2,5DKG by *G. japonicus* NBRC3271 Δ*sldA* strains harboring plasmid pSHO8 (A) or pNK316 (B). The cells were precultivated in 2.5 ml of ΔP medium at 30°C for 24 h and then transferred to 100 ml of the 2,5DKG production medium. Mean values and standard deviations from triplicate experiments are shown. Symbols: open circles, D-glucose; open diamonds, D-gluconate; filled triangles, 2KG; filled squares, 2,5DKG. (C) Refraction index-based elution profile of the culture medium of NBRC3271 Δ*sldA*/pNK316 at 72 h on the ion-exclusion column during HPLC.

2,5DKG, as expected, and the maximum concentration of 97.4 ± 0.30 mM (96% molar yield) was obtained at 60 h. A slight decrease in the amount of 2,5DKG in the fermentation medium of the strain harboring pNK316 was observed; the concentration dropped to 93.6 ± 0.70 mM at 96 h. Such a decrease in 2,5DKG was also seen in *G. oxydans* NBRC3293 (Fig. 2), but it was smaller than that in *G. oxydans* NBRC3293. The biotransformation process driven by the Δ*sldA* strain harboring pNK316 showed the nearly homogeneous production of 2,5DKG (Fig. 5C), making the downstream purification process much easier.

DISCUSSION

We identified the *kgdSLC* genes of 2KGDH in *G. oxydans* NBRC3293 (formerly *Gluconobacter melanogenus* IFO3293). The membrane fractions from the NBRC12528 Δ*adhA* strain harboring pNK316 showed a specific 2KGDH activity of 37.7 ± 1.4 U · (mg protein)$^{-1}$. Heterotrimeric flavoprotein dehydrogenase-cytochrome *c* complexes reported to date show strict substrate specificity: D-gluconate-, D-glucose-, D-sorbitol-, or D-fructose-specific dehydrogenase. In addition to the evidence that *G. oxydans* 2KGDH, the product of the *kgdSLC* genes identified in the present study, also shows strict substrate specificity (13), the phylogenetic analyses suggest that 2KGDH constitutes a new, 5th member in this family as a 2KG-specific enzyme (see Fig. S3 in the supplemental material).

The purified 2KGDH from the recombinant *Gluconobacter* cells showed properties similar to those reported previously (13) and Q-1 reductase activity as well. In addition, another *Gluconobacter* transformant expressing 2KGDH had a 2KG-oxidizing respiratory chain and produced 2,5DKG with a nearly stoichiometric yield. It is reasonable to assume that ubiquinone-10 is a physiological electron acceptor for 2KGDH.

As for bioenergetic properties, an increase in the cell yield was observed in the strain harboring pNK316 compared to that in the reference strain (Fig. 4). Because heterologous expression of *kgdSLC* results in a 2KG oxidation respiratory chain, the proton motive force generated by ubiquinol oxidase may be increased and used for anabolic processes to improve the biomass yield.

We previously reported that *G. oxydans* NBRC3293 produces 2,5DKG from D-glucose via D-gluconate and 2KG (11). In the present study, we found that 2,5DKG is produced as a major metabolite (Fig. 2), but 5KG is also produced in small amounts as a by-product, and considerable amounts of D-gluconate remain in the medium. In contrast, the *G. japonicus* NBRC3271 Δ*sldA* strain consumes D-gluconate completely under the same experimental conditions (Fig. 5A and B). The difference between the D-gluconate consumption profiles of *G. oxydans* NBRC3293 and *G. japonicus* NBRC3271 Δ*sldA* may be the result of the difference in their GADH activities (Table 2). *G. japonicus* NBRC3271 was re-

ported to have at least two types of GADHs (9), whereas the GADH in *G. oxydans* NBRC3293 has not been studied yet. Thus, we are currently analyzing the GADH of *G. japonicus* NBRC3271 and *G. oxydans* NBRC3293 by means of genetic and biochemical methods.

We expressed the *kgdSLC* genes of *G. oxydans* NBRC3293 in *G. japonicus* NBRC3271 Δ*sldA* to achieve the efficient and homogeneous production of 2,5DKG. The *G. japonicus* NBRC3271 Δ*sldA* strain was reported to produce exclusively 2KG, which is the substrate for 2KGDH, from the mixture of D-glucose and D-gluconate (9). The Δ*sldA* strain harboring pNK316 can consume almost all starting materials and produces 2,5DKG as a unique metabolite in a nearly stoichiometric manner. These results demonstrate that the efficient and homogeneous production of 2,5DKG was successfully achieved here via the heterologous expression of *kgdSLC* in the strain *G. japonicus* NBRC3271 Δ*sldA*.

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