Screening of Thermotolerant *Gluconobacter* Strains for Production of 5-Keto-\(\delta\)-Gluconic Acid and Disruption of Flavin Adenine Dinucleotide-Containing \(\delta\)-Gluconate Dehydrogenase

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We isolated thermotolerant *Gluconobacter* strains that are able to produce 5-keto-\(\delta\)-gluconic acid (5KGA) at 37°C, a temperature at which regular mesophilic 5KGA-producing strains showed much less growth and 5KGA production. The thermotolerant strains produced 2KGA as the major product at both 30 and 37°C. The amount of ketogluconates produced at 37°C was slightly less than the amount produced at 30°C. To improve the yield of 5KGA in these strains, we disrupted flavin adenine dinucleotide-gluconate dehydrogenase (FAD-GADH), which is responsible for 2KGA production. Genes for FAD-GADH were cloned by using inverse PCR and an in vitro cloning strategy. The sequences obtained for three thermotolerant strains were identical and showed high levels of identity to the FAD-GADH sequence reported for the genome of *Gluconobacter oxydans* 621 H. A kanamycin resistance gene cassette was used to disrupt the FAD-GADH genes in the thermotolerant strains. The mutant strains produced 5KGA exclusively, and the final yields were over 90% at 30°C and 50% at 37°C. We found that the activity of pyrroloquinoline quinone (PQQ)-dependent glycerol dehydrogenase, which is responsible for 5KGA production, increased in response to addition of PQQ and CaCl₂ in vitro when cells were grown at 37°C. Addition of 5 mM CaCl₂ to the culture medium of the mutant strains increased 5KGA production to the point where over 90% of the initial substrate was converted. The thermotolerant *Gluconobacter* strains that we isolated in this study provide a promising new option for industrial 5KGA production.

*Gluconobacter* is a genus of acetic acid bacteria that are able to oxidize a broad range of sugars, sugar alcohols, and sugar acids, and large amounts of the corresponding oxidized products accumulate in the culture medium. Such “incomplete” oxidation is carried out by membrane-bound enzymes, whose catalytic sites face the periplasm. These enzymes catalyze the oxidation of \(\delta\)-glucose, \(\delta\)-sorbitol, \(\delta\)-mannitol, glycerol, \(\delta\)-gluconate, and the keto- \(\delta\)-gluconates. All of these enzymes are firmly attached to the cytoplasmic membrane, and the electrons abstracted from the substrates are passed on to ubiquinone and then to terminal ubiquinol oxidases, forming simple respiratory chains which create the membrane potential necessary to produce biological energy for these microorganisms.

The oxidation of \(\delta\)-glucose to ketogluconates is known to be catalyzed by a series of enzymes. Pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase oxidizes \(\delta\)-glucose to glucono-\(\delta\)-lactone, and then gluconolactonase converts the glucono-\(\delta\)-lactone to \(\delta\)-gluconate. The formation of ketogluconates in *Gluconobacter* strains has been reported to be catalyzed by two types of membrane-bound glucose dehydrogenases (GADH) (10). One type is flavin adenine dinucleotide (FAD)-GADH, an FAD-containing, 2-keto-\(\delta\)-gluconate (2KGA)-producing enzyme, and the other type is a PQQ-containing, 5-keto-\(\delta\)-gluconate (5KGA)-producing enzyme. The former enzyme has three subunits: an FAD-containing dehydrogenase, a \(c\)-type cytochrome subunit containing three hemes, and a small subunit of unknown function (17). The latter enzyme, which produces 5KGA, is identical to the PQQ-containing polyol dehydrogenase (9), which is known as \(\delta\)-arabitol dehydrogenase (1), \(\delta\)-sorbitol dehydrogenase (20), or PQQ-dependent glycerol dehydrogenase (PQQ-GLDH) (2). PQQ-GLDH has broad substrate specificity but high regio- and stereospecificity, and it catalyzes reactions as predicted by the Bertrand-Hudson rule. This enzyme can oxidize \(\delta\)-gluconate only at the C-5 position to produce 5KGA from \(\delta\)-gluconate; however, the affinity of the enzyme for \(\delta\)-gluconate is quite low. The gene encoding this enzyme was cloned from *Gluconobacter suboxydans* IFO 3255 (11), and two open reading frames (ORFs) were found. One of these ORFs is believed to encode a hydrophobic protein with five membrane-spanning regions, and the other encodes a dehydrogenase subunit similar to that found in several PQQ-dependent enzymes, particularly the PQQ domain of membrane-bound glucose dehydrogenase. In contrast, 2KGA reductase and 5KGA reductase, the NADPH-dependent enzymes located in the cytoplasm, are thought to be involved in gluconate metabolism in the assimilation of 2KGA and 5KGA.

5KGA is a useful raw material for the production of tartaric acid.

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Recently, an FAD-GADH-defective mutant strain of conobacter oxydans 621 H which produced almost exclusively 5KGA from D-glucose was discovered (5). However, the optimization of 5KGA production requires the use of high temperatures and an organic solvent in processing; however, Gray’s method does not.

Most Gluconobacter strains produce both 2KGA and 5KGA from D-gluconate. Thus, production of 5KGA by Gluconobacter species generates 2KGA as a major by-product, and production of the two ketogluconates is competitive in vivo. FAD-GADH-defective mutant strains of Gluconobacter oxydans 621 H which produced almost exclusively 5KGA from D-glucose was discovered (5). However, the optimum temperature for production of 5KGA in this mesophilic strain was around 20°C (19). For cost-effective industrial synthesis of 5KGA, we sought to develop a Gluconobacter strain which is able to produce 5KGA at higher temperatures, such as 37°C, in order to reduce the cost of cooling during fermentation.

We successfully isolated thermotolerant Gluconobacter strains that are able to produce 5KGA at 37°C. We cloned the FAD-GADH gene and constructed FAD-GADH-defective mutants that produced almost exclusively 5KGA from D-gluconate at both ambient temperatures and higher temperatures up to 37°C. We believe that the thermotolerant strains reported in this study should be useful for industrial 5KGA production.

MATERIALS AND METHODS

Materials. 2-Ketogluconic acid (hemiacetal salt) and 5-ketogluconic acid (potassium salt) were purchased from Sigma (St. Louis, MO). Yeast extract was a kind gift from Oriental Yeast (Tokyo, Japan). All other chemicals were obtained from commercial sources. The oligonucleotides used in this study were purchased from FASMAC Co. Ltd. (Kanagawa, Japan) and are listed in Table 1.

Bacterial strains and growth conditions. All Gluconobacter strains were maintained on potato agar slants (20 g glucose, 5 g sucrose, 10 g yeast extract, 10 g peptone, and 15 g agar; pH 7.0) and on potato plates (1% sorbitol, 1% glycerol, 0.3% polypeptone, 0.3% yeast extract, and 2% agar; pH 7.0). Then, 200 ml of medium from a culture of each of the remaining isolates was reacted with 1 ml of Resorcinol reagent (49 ml 0.5% [w/v] sulfuric acid, 10 g sodium carbonate, 10 ml of Resorcinol reagent, 168 ml of concentrated HCl, 273 ml of distilled water) at 80°C for 20 min. The product of the reaction of D-glucose with Resorcinol reagent was a red compound, whereas the reaction with 5KGA generated a dark brownish green precipitate. D-Gluconate and D-glucose with Resorcinol reagent was a red compound, whereas the reaction with 5KGA generated a dark brownish green precipitate. D-Gluconate and D-glucose with Resorcinol reagent was a red compound, whereas the reaction with 5KGA generated a dark brownish green precipitate. D-Gluconate and D-glucose with Resorcinol reagent was a red compound, whereas the reaction with 5KGA generated a dark brownish green precipitate.

Screening of thermotolerant 5KGA-producing Gluconobacter strains. All Gluconobacter strains used in the screening experiment were isolated from various sources in Thailand (12) and maintained on potato agar slants. For rough screening, a single colony of each isolate was inoculated into 2% glucose-gluconate medium (2% G-GA water [final volume, 1 liter]) was used to screen for 5KGA-producing strains. To measure production of 5KGA, 2% glucose-2% gluconate medium (2% G-GA medium) was used. 2KGA were not found to generate any visible reaction products with Resorcinol.

Preparation of membrane fractions. The membrane fractions were prepared as described previously (22) and suspended in 100 mM acetate buffer (pH 5.0).

### Table 1. Bacterial strains, plasmids, and primers used in this study

<table>
<thead>
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<th>Strain, plasmid, or primer</th>
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<th>Source or reference</th>
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<td>Km', strain THE42 with gndG disrupted</td>
<td>This study</td>
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<td>Km', strain THG42 with gndG disrupted</td>
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<td>Contains nonpolar Km' cassette</td>
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<td>1.9-kb PCR product inserted into pGEM-T Easy</td>
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Holomeration of PQQ-GLDH. The protein concentrations of the membrane fractions suspended in acetate buffer (pH 5.0) were adjusted to 10 mg/ml by addition of 15 μM PQQ and 10 mM CaCl2. Samples were then incubated on ice overnight to obtain the fully holomerized form of PQQ-GLDH before the enzyme assay was performed.

Enzyme assay. All measurements were obtained at 25°C. Enzyme activity was measured using potassium ferricyanide as the electron acceptor. Each reaction mixture (0.9 ml), containing 0.1 M substrate, McIlvaine buffer (pH 5.0), and the appropriate amount of enzyme solution (2 to 20 μl of a suspension), was preincubated for 5 min, and then 0.1 ml of 0.1 M potassium ferricyanide was added and rapidly mixed to start the reaction. After incubation for a defined period, the reaction was stopped by addition of 0.5 ml DNP reagent [0.3% (wt/vol) Fe2(SO4)3, 8.1% (wt/vol) phosphoric acid, 0.3% (wt/vol) sodium dodecyl sulfate]. After incubation for 20 min to stabilize the color, the mixture was diluted with 3.5 ml of distilled water to obtain a total volume of 5.0 ml, and the absorbance at 660 nm was measured with a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of substrate per min. The phenol methanol method (PMS) reductase activity of the membrane fraction was measured spectrophotometrically by coupling with 2,6-dichlorophenolindophenol (DCIP) at 25°C. The reduction of oxidized DCIP was measured by determining the decrease in absorbance at 600 nm. Each reaction mixture (1 ml) contained 100 mM substrate, 0.2 mM PMS, 0.11 mM DCIP, 1.2 mM NaNO3, the appropriate amount of enzyme (2 to 20 μl of a suspension), and McIlvaine buffer (pH 5.0). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of substrate per min under these assay conditions. The extinction coefficient of DCIP at pH 5.0 is 3.9 × 104 M−1 cm−1.

Measurement of protein concentrations. Protein concentrations were measured using a modification of the Lowry method (4). Bovine serum albumin was used as the standard protein.

Measurement of 2KGA and 5KGA. 2KGA and 5KGA were enzymatically quantified by using 2KGA reductase and 5KGA reductase, respectively (14). Qualitative analysis of 2KGA and 5KGA was performed using thin-layer chromatography (TLC). Samples were spotted on a Silica Gel 60 plate (Merck, Darmstadt, Germany) and developed with a solvent reagent containing ethyl acetate, acetic acid, methanol, and deionized water at a ratio of 6:1.5:1.5:1. After development, the plate was dried at 120°C for 10 to 20 min for color development. The 2KGA and 5KGA regions were colored yellow with a solvent reagent containing ethyl acetate, acetic acid, methanol, and 15% phosphoric acid.

Measurement of protein concentrations. Protein concentrations were measured using a modification of the Lowry method (4). Bovine serum albumin was used as the standard protein.

RESULTS

Screening of thermotolerant 5KGA-producing Gluconobacter strains. A single colony of each of 84 isolates from natural sources in Thailand (12) was inoculated into 2% glucose-glucuronate medium to test for the production of 5KGA and incubated for 48 h at 30°C and for 60 h at 37°C. Strains that accumulated 2,5-diketogluconic acid (2,5DKG) in the culture medium, as determined by the production of a brown compound, were eliminated because 2,5DKG is a product of 2KGA oxidation which is catalyzed by the membrane-bound FAD–2-ketogluconate dehydrogenase found in some Gluconobacter strains; therefore, formation of 2,5DKG was expected to be the result of formation of high levels of 2KGA and thus low levels of 5KGA in these strains. A total of 20 isolates were eliminated in this step. Of the 64 remaining isolates, 29 accumulated 5KGA at both 30 and 37°C, as determined by the formation of a brownish green precipitate after reaction with the Resorcinol reagent. We then compared the amounts of 5KGA accumulated by these strains by using 16-h preculures into 2% glucose-glucuronate medium and incubating them for 36 h at 30°C and for 48 h at 37°C. Shorter incubation times were used in this step to determine the amount of 5KGA produced and the amount of glucose remaining in the earlier growth phase. Of the 29 isolates obtained, 3 (THE42, THF55, and THG42) were able to accumulate large amounts of 5KGA at 37°C, a temperature at which regular mesophilic strains, such as G. suboxydans IFO 12528, neither grow well nor produce 5KGA, although they produced more 5KGA at 30°C than at 37°C (see below).

Characterization of thermotolerant Gluconobacter strains. The formation of 5KGA by the three thermotolerant strains selected was confirmed by TLC, as shown in Fig. 1. All three strains formed both 2KGA and 5KGA, which is typical for Gluconobacter strains, at both 30 and 37°C. The formation of 2KGA and 5KGA was confirmed by reaction with the specific enzymes 2KGA reductase and 5KGA reductase.

The growth and production profiles of the three strains were examined in 2% G-GA medium together with those of a type 5KGA-producing strain, G. suboxydans IFO 12528. The growth yields of the thermotolerant strains were similar, and in all three strains 2KGA was the major product (see Fig. 6). When approximately 5 kb and 7 kb long were obtained from the SacII and EcoRI religated fragments, respectively. The remaining part of the gene encoding the cytochrome c subunit and the downstream region was then obtained using an in vitro cloning strategy.
G. suboxydans IFO 12528 was cultured at 30°C for 48 h, the growth yield was lower (222 Klett units), and this strain produced a higher proportion of 5KGA (148 \mu M standard solutions (lane 1, \( \delta \)-glucose; lane 2, sodium gluconate; lane 3, 2KGA; lane 4, 5KGA); lane 5, 3 \mu l of culture medium (before inoculation); lanes 6 to 8, 3 \mu l of culture medium after growth at 30°C (lane 6, THE42; lane 7, THF55; lane 8, THG42); lanes 9 to 11, 4 \mu l of culture medium after growth at 37°C (lane 9, THE42; lane 10, THF55; lane 11, THG42).

Cloning and sequence analysis of FAD-GADH genes from isolated thermotolerant strains. The genes encoding FAD-GADH were cloned as described in Materials and Methods. We sequenced over 6 kb of DNA from each strain, which covered the three ORFs predicted to contain the gene for FAD-GADH and three ORFs encoding hypothetical proteins. The DNA sequences obtained for the thermotolerant strains were identical, and a map of the fragments obtained from strain THF55 is shown in Fig. 2. We designated these ORFs ORF-1, ORF-2, gndF, gndG, gndH, and ORF-3, and they are similar to the GOX1234, GOX1233, GOX1232, GOX1231, GOX1230, and GOX1229 genes, respectively, identified in the G. oxydans 621 H genome (13). The gndFGH ORFs exhibited 76%, 89%, and 68% identity to GOX1232, GOX1231, and GOX1230, respectively.

The putative amino acid sequence encoded by gndF was shown to contain a tat-dependent signal sequence (3) at the N terminus. The amino acid sequence of GndF also showed high levels of identity to several protein sequences in databases, including 59% identity to the sequence of a putative gluconate 2-dehydrogenase subunit 3 precursor in Gluconacetobacter diazotrophicus PA15 (accession no. ZP_02981641), 58% identity to the sequence of a gluconate 2-dehydrogenase in Burkholderia multivorans 17616 (accession no. ABX18062), and more than 45% identity to the sequences of several gluconate 2-dehydrogenases annotated for the genomes of Burkholderia, Pseudomonas, and Erwinia species. GndF also showed 44% identity to GndS in G. dioxyacetonicus IFO 3271 (21), which was recently reported to encode the small subunit of FAD-GADH.

The putative amino acid sequence encoded by gndG included a glycine box (GXGXXG), which is a motif for FAD binding in the N-terminal part of the sequence and which is similar to motifs in several FAD-containing enzymes. GndG has a conserved domain sequence found in members of the glucose-methanol-choline oxidoreductase family, which is a protein family whose members bind FAD as a cofactor. It also showed 61% identity to GndL of G. dioxyacetonicus IFO 3271 (21).

GndH had three heme c-binding motifs (CXXCH), C_{43}AA CH_{47}, C_{193}GACH_{198} and C_{330}AACH_{334}, which is similar to many cytochrome c subunits of membrane-bound enzymes found in Gluconobacter strains, such as alcohol dehydrogenase, sorbitol dehydrogenase, and aldehyde dehydrogenase. It also showed 44% identity to G. dioxyacetonicus IFO 3271 GndC (21).

Construction of FAD-GADH-defective mutants of thermotolerant Gluconobacter strains. As the isolated thermotolerant Gluconobacter strains competitively produced 2KGA as a major product along with 5KGA, we disrupted their FAD-GADH genes by inserting a nonpolar kanamycin resistance cassette into gndG to eliminate 2KGA production in these strains. Several kanamycin-resistant colonies which did not have resistance to ampicillin were obtained, indicating that there was disruption by a double crossover, and this was confirmed by PCR with specific primers (Fig. 3). We produced gndG::Km mutants of the THE42, THF55, and THG42 strains. All three mutants showed no 2KGA production as determined by TLC.

FIG. 1. 2KGA and 5KGA production in culture medium by the thermotolerant Gluconobacter strains isolated. Cells were grown in 2% G-GA medium at 30°C for 2 days or at 37°C for 3 days. The culture supernatants were analyzed by TLC as described in Materials and Methods. Lanes 1 to 4, 4 \mu l of culture medium after growth at 30°C (lane 1, THE42; lane 2, G. suboxydans IFO 12528 was cultured at 30°C for 48 h, the growth yield was slightly lower and the level of ketogluconate produced was lower at this temperature than at 30°C. (see Fig. 6).

FIG. 2. Schematic diagram of the gene fragment obtained in this study. PCR primers are indicated by arrows. The open triangle indicates the position where the kanamycin resistance gene cassette was inserted for mutation. ORF-3 is not complete.
analysis (Fig. 4), indicating that gndF, gndG, and gndH are the genes responsible for 2KGA production in these thermotolerant strains.

We then measured membrane-bound enzyme activity in the mutants. PMS reductase activity was observed even in the absence of substrate for the membrane fractions prepared from cells grown at 30°C. The autoreduction was due to high alcohol dehydrogenase activity with a trace amount of contaminating ethanol in the assay mixture, not to the substrate remaining from the culture medium. Thus, in this case the activity was manifested only as ferricyanide reductase activity. The total enzyme activity with D-arabitol and D-gluconate. The total enzyme activity with D-arabitol and D-glucuronate is thought to result from FAD-GADH and PQQ-GLDH activity, whereas the enzyme activity with D-arabitol after incubation with deionized distilled water overnight; black bars, activity with D-arabitol after incubation with 15 μM PQQ and 10 mM CaCl₂ overnight; open bars, activity with D-gluconate after incubation with deionized distilled water overnight; striped bars, activity with D-gluconate after incubation with 15 μM PQQ and 10 mM CaCl₂ overnight. Assays were performed in triplicate; the bars indicate averages, and the error bars indicate standard deviations. WT, wild type.
hydrogenase (which was about 1/10 the expression in the cells grown at 30°C), whose cytochrome c subunit is known to mediate the transfer of electrons from membrane-bound glucose dehydrogenase to ferricyanide via ubiquinone (18). PQQ-GLDH is also known to react with ubiquinone-2 (1). Thus, enzyme activity was measured with PMS-DCIP as the electron acceptor (Fig. 5b). Although we could not compare enzyme activities quantitatively because we used different assay systems for cells grown at 30°C and cells grown 37°C, both the D-arabitol and D-gluconate dehydrogenase activities appeared to be lower in cells grown at 37°C than in cells grown at 30°C. The D-arabitol dehydrogenase activities were similar in the wild-type and mutant strains, whereas the D-gluconate dehydrogenase activity was decreased in the mutant strains. When cofactors were added, the D-arabitol dehydrogenase activity in all strains and the D-gluconate dehydrogenase activity in the mutant strains more than doubled, indicating either that POQ-GLDH easily releases its cofactors or that production of PQQ is not sufficient when cells are grown at 37°C.

Production of 5KGA by FAD-GADH-defective mutants. Mutant strains were cultured in 2% G-GA medium at 30 and 37°C, and the accumulation of 2KGA and 5KGA was monitored during the course of cultivation (Fig. 6). The growth of all strains at 37°C was slightly slower than that at 30°C. At 30°C, all three mutant strains converted substrates to primarily 5KGA, and trace amounts of 2KGA were also produced. The conversion yield was over 90% of the initial amount of the substrate (D-glucose and D-gluconate) after 60 h of cultivation. Assimilation of 2KGA and 5KGA was also observed after this period, similar to what was observed for wild-type strains. At 37°C, the mutant strains also produced 5KGA along with trace amounts of 2KGA; however, the production yield was only one-half that at 30°C. The amount of 5KGA produced by the mutant strains was about twice the amount produced by the wild-type strains. The final conversion yield after 96 h of cultivation was about 50% of the initial amount of the substrates.

Improved 5KGA production at higher temperatures. As the POQ-GLDH activity in the wild-type and mutant strains grown at 37°C was dependent on addition of cofactors (POQ and/or Ca²⁺) (Fig. 5), it was expected that addition of Ca²⁺ to the culture medium would improve production. Addition of CaCl₂ at concentrations up to 5 mM had little effect on growth, and the conversion yield increased to about 90% of the initial amount of the substrate (Fig. 7). When CaCl₂ was added at concentrations higher than 5 mM, growth was somewhat inhibited and 5KGA production was reduced. These results indicate that addition of CaCl₂ improves 5KGA production at 37°C and that the optimum concentration is around 5 mM.

**DISCUSSION**

We isolated three thermotolerant *Gluconobacter* strains that are able to produce 5KGA at 37°C, which the mesophilic strains could not do, although these thermotolerant strains still produced 2KGA as the major product. We did not find a naturally occurring strain that produced significantly more 5KGA than 2KGA in this study. Recently, it was shown that disruption of the FAD-GADH gene, which is responsible for 2KGA production in *G. oxydans* 621 H (which is genetically identical to *G. suboxydans* IFO 12528), eliminated 2KGA formation, leading to production of only 5KGA (5), although *G. suboxydans* IFO 12528 itself has previously been shown to produce 5KGA as the major product along with a small amount of 2KGA as a by-product (19). Thus, we disrupted the FAD-GADH gene in *Gluconobacter*, *gndG*, and the disruption mutants produced almost exclusively 5KGA. Furthermore, we found that addition of calcium chloride to the culture medium increased 5KGA production. The newly isolated strains reported in this study provide an alternative method for 5KGA production at higher temperatures.

In this study, we cloned the genes for FAD-GADH and its flanking regions from the thermotolerant strains. The gene sequences obtained are quite similar to the corresponding region (GOX1234 to GOX1229) in the genomic sequence of *G. oxydans* 621 H (13) and are much more similar than the *gndSLC* gene of *G. dioxyaceticus* IFO 3271 (21). Recently, another FAD-GADH gene was identified in *G. dioxyaceticus* IFO 3271, and it is quite similar to GOX1232 to GOX1230 (unpublished results). Therefore, the FAD-GADH genes cloned from the thermotolerant strains in this study are designated *gndFGH* and not *gndSLC*. We did not determine whether *gndSLC* are present in the thermotolerant strains, but it is likely that they do not, because the gluconate dehydrogenase activity found in the membrane fractions of the mutants defective in *gndG* seemed to be a result of only POQ-GLDH activity (Fig. 5), and the mutants produced almost exclusively 5KGA (Fig. 6), which is quite different than the situation in *G. dioxyaceticus* IFO 3271 (21).

We constructed FAD-GADH-defective mutants of the thermotolerant strains by insertion of a kanamycin resistance cassette at the site of *gndG*. The mutant strains grew like the wild-type strains, which was not the case when the mutations were in *G. oxydans* 621 H, whose mutants had a longer lag period before the oxidation of D-glucose (5). Nonetheless, as reported previously for *G. oxydans* 621 H (5), the production of 5KGA by the thermotolerant FAD-GADH-defective mutant strains was significantly greater than that by wild-type strains at both 30°C and 37°C. At 30°C, the mutants produced 5KGA from more than 90% of the initial substrate (D-glucose plus D-gluconate) until around 60 h during cultivation, and then production slowly decreased. However, at 37°C, only about 50% of the initial substrate was converted to 5KGA after 96 h, but the amount increased continuously (Fig. 6). Wild-type thermotolerant strains also showed a decrease in 5KGA and 2KGA production at 30°C but not at 37°C; therefore, it is possible that these strains are unable to assimilate both 2KGA and 5KGA at 37°C. The enzymes responsible for assimilation of 2KGA and 5KGA (2KGA and 5KGA reductases) were found to be stable at 37°C (data not shown); therefore, we believe that the 2KGA and 5KGA transporter proteins may be inactive at higher temperatures, leading to decreased assimilation of 2KGA and 5KGA at 37°C.

The POQ-GLDH activity responsible for 5KGA production in the membrane fraction was quite low at 37°C, and the activity increased more than twofold after incubation with POQ and calcium chloride (Fig. 5), indicating that more than one-half of the POQ-GLDH was in the apo form. This is probably due to instability of POQ-GLDH at 37°C, which decreases the affinity of POQ and the calcium ion. Addition of calcium chloride to the culture medium increased the stability
FIG. 6. Production of ketogluconates by wild-type and mutant strains. (a) Wild-type and mutant strains grown in 2% G-GA medium at 30°C. (b) Wild-type and mutant strains grown in 2% G-GA medium at 37°C. Gray bars, 2KGA concentration; black bars, 5KGA concentration. 2KGA and 5KGA were measured enzymatically as described in Materials and Methods. WT, wild type.
of PQQ-GLDH and improved 5KGA production at 37°C. The optimum concentration for 5KGA production was around 5 mM, although cell growth was slightly inhibited as the concentration of calcium chloride was increased (Fig. 7). Under optimum conditions, over 90% of the initial substrate was converted to 5KGA within 3 days.

The thermotolerant strains isolated in this study grew and produced 5KGA at temperatures up to 37°C, a temperature at which mesophilic strains neither grow nor produce any product. These characteristics make the mutant thermotolerant Gluconobacter strains isolated in our study highly useful for industrial 5KGA production.

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