Engineering of *Corynebacterium glutamicum* with an NADPH-Generating Glycolytic Pathway for L-Lysine Production\(^\text{V}\)

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A sufficient supply of NADPH is a critical factor in l-lysine production by *Corynebacterium glutamicum*. Endogenous NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of *C. glutamicum* was replaced with nonphosphorylating NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (GapN) of *Streptococcus mutans*, which catalyzes the reaction of glyceraldehyde 3-phosphate to 3-phosphoglycerate with the reduction of NADP\(^+\) to NADPH, resulting in the reconstruction of the functional glycolytic pathway. Although the growth of the engineered strain on glucose was significantly retarded, a suppressor mutant with an increased ability to utilize sugars was spontaneously isolated from the engineered strain. The suppressor mutant was characterized by the properties of GapN as well as the nucleotide sequence of the gene, confirming that no change occurred in either the activity or the basic properties of GapN. The suppressor mutant was engineered into an l-lysine-producing strain by plasmid-mediated expression of the desensitized *lysC* gene, and the performance of the mutant as an l-lysine producer was evaluated. The amounts of l-lysine produced by the suppressor mutant were larger than those produced by the reference strain (which was created by replacement of the preexisting gapN gene in the suppressor mutant with the original gapA gene) by ~70% on glucose, ~120% on fructose, and ~100% on sucrose, indicating that the increased l-lysine production was attributed to GapN.

These results demonstrate effective l-lysine production by *C. glutamicum* with an additional source of NADPH during glycolysis.

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On the other hand, nonphosphorylating NADP-dependent glyeraldehyde 3-phosphate dehydrogenase (GapN; EC 1.2.1.9), which catalyzes the irreversible oxidation of glyeraldehyde 3-phosphate to 3-phosphoglycerate with the reduction of NADP⁺ to NADPH, is found in photosynthetic eukaryotes (10, 21) and in some Gram-positive bacterial species, such as Streptococcus (1, 4, 6, 9) and Clostridium species (8). In this work, we attempted to use the heterologous Streptococcus mutans gapN gene to engineer C. glutamicum. Our objectives were (i) to utilize the GapN enzyme as an additional source of NADPH during glycolysis and (ii) to lead the engineered strain to an effective L-lysine-producing strain.

**Materials and Methods**

**Bacterial strain, growth conditions, and plasmids.** The wild-type C. glutamicum strain ATCC 13032 was used in this study. Complete medium BY (32) and minimal medium MM (32) were used for the cultivation of ATCC 13032 and its derivative mutants. For the growth test, liquid culture using MM was done in L-type test tubes shaken on a Monod shaker at 48 strokes/min. LFG1 medium (24) was also used for the growth test and for L-lysine production; this medium was done at 30°C in test tubes reciprocally shaken at 120 strokes/min.

**Strain construction.** The sequences of the primers used in this study are listed in Table 1. All primers were designed based on the genomic sequences of C. glutamicum (AE014133), which are publicly available at http://gib.genes.nig.ac.jp/single/index.php?spid=Cglu_ATCC13032 and http://gib.genes.nig.ac.jp/single/index.php?spid=Smut_AA159, respectively.

**For the chromosomal deletion of the gapA gene, plasmid pCgapA, containing the internally deleted gapA gene, was constructed as follows.** The 5’ region of the gapA gene was amplified by PCR using two primers, gapAup5 and gapAdn3, according to the following program: heating to 94°C for 5 min; 25 cycles, each consisting of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and then extension for 3 min at 72°C. The resulting 1.8-kb fragment was digested with BamHI and then ligated to BamHI-digested pCS299P. Recombinant DNA techniques. Standard protocols (30) were used for the construction, purification, and analysis of plasmid DNA and for the transformation of E. coli. Chromosomal DNA was extracted from C. glutamicum and S. mutans by the methods described by Saio and Miura (29) and those described by Schroder et al. (31), respectively. Transformation of C. glutamicum by electroporation was carried out by the method described by van der Rest et al. (33), using the Gene Pulser II electroporation system (Bio-Rad Laboratories, Inc., Hercules, CA). PCR was performed using a DNA thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA), using Pyrobest DNA polymerase (Takara Bio, Inc., Shiga, Japan). Each of these programs will be described in greater detail in subsequent sections. Sequencing for confirmation of the nucleotide sequences of relevant DNA regions was performed using an ABI PRISM 377 DNA sequencer from Applied Biosystems, with an ABI PRISM BigDye Terminator cycle sequencing kit (Perkin Elmer). The subsequent electrophoresis analysis was carried out by Pageset SQC-5ALN 377 (Toyobo, Japan).

**Table 1. Sequences of primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gapAup5</td>
<td>5’-TAGTGATCAGTGGCACTCCGGCAAAACC-3’</td>
</tr>
<tr>
<td>gapAN3</td>
<td>5’-AATCGGACGGTGTAAGGCCCAATCTACCTCAGATGCGT-3’</td>
</tr>
<tr>
<td>gapAC5</td>
<td>5’-ACGATCTCGAGGTAGTGTTGAGGCGCTACACTGAGCT-3’</td>
</tr>
<tr>
<td>gapAdm3</td>
<td>5’-TGATGATACATCTCGGTTCATCAAGCAGTGGC-3’</td>
</tr>
<tr>
<td>gapBup5</td>
<td>5’-CAATGATACATCTTGATCGACCTAACAGTTTC-3’</td>
</tr>
<tr>
<td>gapBN3</td>
<td>5’-GACCTGGTGGAGTACGCGCAAATTTTGCGACCACTAGGCGC-3’</td>
</tr>
<tr>
<td>gapBC5</td>
<td>5’-GGCGCATCTGTTGCGCAAAATGGTCTCCTAAAGATGTAG-3’</td>
</tr>
<tr>
<td>gapBdn3</td>
<td>5’-TTTCGAGATCTGGAAGTGTTCCGCAACAAAAGATGTAG-3’</td>
</tr>
<tr>
<td>CGLgapAupXB</td>
<td>5’-GGATCCGGTAAAGTGTTCCGCAACAAAAGATGTAG-3’</td>
</tr>
<tr>
<td>CGLgapAdn3</td>
<td>5’-GGATCCGGTAAAGTGTTCCGCAACAAAAGATGTAG-3’</td>
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<tr>
<td>CGLgapBupXB</td>
<td>5’-GGATCCGGTAAAGTGTTCCGCAACAAAAGATGTAG-3’</td>
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<tr>
<td>CGLgapBdn3</td>
<td>5’-GGATCCGGTAAAGTGTTCCGCAACAAAAGATGTAG-3’</td>
</tr>
<tr>
<td>reLysC311F3</td>
<td>5’-TTTGTTAGCTCGAACAGTGTAATGAATAGCAGTGTAATGAATAGCAGTGTAAT-3’</td>
</tr>
<tr>
<td>reLysC311R3</td>
<td>5’-TTTGTTAGCTCGAACAGTGTAATGAATAGCAGTGTAATGAATAGCAGTGTAAT-3’</td>
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<tr>
<td>reLysC311R5</td>
<td>5’-TTTGTTAGCTCGAACAGTGTAATGAATAGCAGTGTAATGAATAGCAGTGTAAT-3’</td>
</tr>
</tbody>
</table>

*Bell sites are underlined, and BamHI sites are italicized.*

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The gapB gene was amplified by PCR using two primers, gapBup5 and gapBN3, with the wild-type ATCC 13032 genomic DNA as a template. Similarly, the 3′ region of the gene was amplified using gapBCS and gapBDn3. Each of these reactions was performed according to the following program: heating to 94°C for 5 min; 25 cycles, each consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and then extension for 3 min at 72°C, which amplified the 750-bp and the 850-bp fragments, respectively. As the two primers gapBN3 and gapBCS were complementary to each other, fusion PCR was performed using the two resulting purified fragments as templates and the primers gapBup5 and gapBDn3, according to the following program: heating to 94°C for 5 min; 25 cycles, each consisting of 94°C for 30 s and 70°C for 2 min; and then extension for 3 min at 70°C. The resulting 1.6-kb fragment contained the deleted gapB gene, which was shortened by in-frame deletion of the inner sequence. The fragment was digested with BclI and then ligated to BamHI-digested pESB30 to yield pCgapB. Defined chromosomal gapA deletion was designated strain GPN. This strain showed no growth on either glucose or acetate, confirming that no other GAPDH isozymes are present in this organism.

Preparation of soluble fraction and enzyme assays. C. glutamicum strains were grown to late log phase in 200 ml of MM liquid medium containing 1% (wt/vol) glucose in 2-liter flasks reciprocally shaken at 120 strokes/min. Cells were collected by centrifugation at 10,000 × g for 10 min and then washed once with 50 mM triethanolamine hydrochloride (pH 8.0) containing 1 mM EDTA and 1 mM dithiothreitol. The cells were suspended in 4 volumes of the same buffer and sonicated on ice for 5 min using a UD-200 ultrasonic disruptor (Tomy Seiko Co., Ltd., Tokyo, Japan). Cell debris was removed by centrifugation at 10,000 × g for 30 min, and the supernatant was further ultracentrifuged at 100,000 × g for 60 min using an Optima TL ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA). The resulting supernatant was used for the enzyme assays. All steps were done at 4°C unless otherwise stated. GAPDH activity was spectrophotometrically measured according to the method described by Omumasaba et al. (27) using a Shimadzu MultiSpec-1500 spectrophotometer (Shimadzu Co., Kyoto, Japan). Nonphosphorylating NADP-dependent GapN activity was measured according to the method described by Crow and Wittenberger (6). One unit of activity is defined as 1 μmol of NADP(H) formed per min.

Analysis. The bacterial growth was monitored by measuring the optical density at 600 nm (OD600) of the culture broth with a Miniphoto 518R spectrophotometer (Taisei, Saitama, Japan). Glucose concentration was determined according to the method described by Ohnishi et al. (26). L-Lysine HCl according to the method described by Ohnishi et al. (26). Fructose and sucrose concentrations were determined using a sucrose/D-glucose/D-fructose F kit (Roche Diagnostics, Basel, Switzerland). Protein content was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories).

RESULTS

Roles of the two C. glutamicum GAPDHs in carbon metabolism. C. glutamicum has two GAPDHs, GapA and GapB. We obtained the corresponding gene deletion mutants (the ΔgapA and ΔgapB strains) from wild-type ATCC 13032 and compared their abilities to utilize carbon sources using a minimal medium, MM (Fig. 1). The ΔgapA strain exhibited no growth on glucose and impaired growth on acetate. The gapB disruption appeared to have scarcely any effect, irrespective of the carbon source tested. These results indicate that GapA is indispensable to glycolysis and also involved in gluconeogenesis but that GapB is responsible only for gluconeogenesis. These results agree with the findings obtained by Omumasaba et al. (27) with the use of another wild-type strain, C. glutamicum R. We also constructed the ΔgapAB strain, a double-deleted mutant. This strain showed no growth on either glucose or acetate, confirming that no other GAPDH isozymes are present in this organism. All mutants showed comparable levels of growth on the complete medium (BY).

Expression of the S. mutans gapN gene in C. glutamicum. We attempted to create a new step allowing NADPH supply in the glycolytic pathway of C. glutamicum using a GapN enzyme from S. mutans. For this purpose, the heterologous gapN gene was amplified by PCR using two primers, gapNup5 and gapNBdown, with the wild-type ATCC 13032 genomic DNA as a template. The resulting 1.6-kb fragment contained the deleted gapN gene, which was shortened by in-frame deletion of the inner sequence. The fragment was digested with BclI and then ligated to BamHI-digested pESB30 to yield pCgapN. Defined chromosomal gapA deletion was designated strain GPN. This strain showed no growth on either glucose or acetate, confirming that no other GAPDH isozymes are present in this organism.

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was introduced into the ΔgapAB strain so that the deleted gapA gene was replaced with the full-length gapN gene. This experimental setup should reveal the effects of GapN on sugar metabolism in a straightforward manner, because no intrinsic GAPDHs are present in the ΔgapAB strain. The introduction of the gapN gene conferred the ability to grow on the MM medium with glucose on the double-deleted mutant (Fig. 2). On the other hand, the resulting strain, GPN, failed to grow on acetate (data not shown). These results indicate that this sugar catabolism occurred by a modified glycolytic pathway reconstructed by the irreversible GapN. A soluble fraction prepared from strain GPN grown on the same MM medium with glucose exhibited a comparably moderate level of NADP-dependent GapN activity (Table 2). Taken together, these results led us to conclude that the engineered strain performed the glycolytic process via GapN accompanying the generation of NADPH. However, both a prolonged lag phase and a lower growth rate compared to the levels for the wild-type and ΔgapB strains were observed in strain GPN (Fig. 2).

Isolation of spontaneous mutants with improved growth on glucose. Judging from the insufficient growth, as shown in Fig. 2, strain GPN is likely to be a less advantageous host for l-lysine production than the other strains tested. Unfortunately, this disadvantage was more marked when the strain was grown on fermentation medium LFG1 containing 5% glucose for reasons not understood (Fig. 3). For the initial 5 days, strain GPN did not show significant growth, like the ΔgapAB strain, which was unable to utilize glucose. However, after a long lag phase, explosive growth was observed in the culture, suggesting that suppressor mutants which had acquired an increased ability to grow on the glucose-based medium appeared spontaneously. Forty candidates were isolated from the broth at the end of the culture and classified into several groups according to their growth rates on glucose (data not shown). Among these candidates, mutant RE2 showed the most improvement in growth rate on MM and especially on LFG1 medium (Fig. 2 and 3) and was therefore used for further analysis. No growth on acetate was observed in mutant RE2 (data not shown), which is reasonably explained by the irreversible reaction of the GapN enzyme. We also examined whether a fast grower similar to RE2 appeared when an outgrowth culture of strain GPN was used to inoculate fresh MM medium or in MM medium.

![FIG. 2. Growth of the gapN-expressing strain.](image1)

![FIG. 3. Growth of the gapN-expressing strain and generation of a spontaneous mutant from the recombinant strain.](image2)

### TABLE 2. Specific activities of GAPDH and GapN in recombinant C. glutamicum strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>GADH activity (mU/mg)</th>
<th>GapN activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAD⁺</td>
<td>NADP⁺</td>
</tr>
<tr>
<td>ATCC 13032</td>
<td>439.7 ± 19.3</td>
<td>Tr amt⁺</td>
</tr>
<tr>
<td>ΔgapB strain</td>
<td>430.1 ± 21.2</td>
<td>-</td>
</tr>
<tr>
<td>GPN</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>RE2</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Data represent mean values and corresponding standard deviations obtained using the soluble fraction from three independent cultures. - , not detected; ND, not determined.

b~1.0 mU/mg.
gene, revealing that no mutation that could affect the expression of the *gapN* gene or the enzymatic properties of GapN was present in these regions. Thus, strain RE2 was a host strain that fulfilled our requirements.

**1-Lysine production by strain RE2.** We evaluated the performance of strain RE2 as a host for 1-lysine production. For this purpose, plasmid pCAK311 ([yC(T311I)], which could confer overproduction of 1-lysine on a wild-type strain, was introduced into strain RE2, its parental strain GPN, the Δ*gapB* strain, and wild-type ATCC 13032. The resulting plasmid carriers were cultivated in LFG1 medium containing 3% glucose, fructose, and sucrose at 30°C with reciprocal shaking at 120 strokes/min for 4 days. Values are means of replicated cultures, which showed <5% differences between each other. The standard deviations from the means are indicated as error bars. 1-Lysine concentrations on glucose (white column), on fructose (gray column), and on sucrose (dotted column) are shown. ■ growth.

**FIG. 4.** 1-Lysine production and growth levels of *C. glutamicum* strains on various sugars. Wild type *C. glutamicum*, the Δ*gapB* strain, strain GPN, suppressor mutant RE2, and strain RE2A were cultivated in 5 ml of LFG1 medium supplemented with 3% glucose, fructose, and sucrose at 30°C with reciprocal shaking at 120 strokes/min for 4 days. Values are means of replicated cultures, which showed <5% differences between each other. The standard deviations from the means are indicated as error bars. 1-Lysine concentrations on glucose (white column), on fructose (gray column), and on sucrose (dotted column) are shown. ■ growth.

![Graph showing 1-Lysine production and growth levels of *C. glutamicum* strains on various sugars.](http://aem.asm.org/)

**DISCUSSION**

In this study, replacement of the native NADP-dependent GAPDH with the NADP-dependent GapN protein from *S. mutans*, followed by isolation of a suppressor mutant with improved growth, led to considerably increased production of 1-lysine in *C. glutamicum*. This not only reconfirms the importance of NADPH supply in efficient 1-lysine production but also demonstrates that our approach allows reconstruction of a functional glycolytic pathway with a new route of NADPH supply in *C. glutamicum*. Since carbon of sugar inevitably passes through the reaction of GapN even if it flows via glycolysis or via the PPP, the engineered strain can generate more NADPH than the wild-type strain, which is clearly reflected by the results of 1-lysine production. Considering that 1 mol of 1-lysine requires 4 mol of NADPH for its biosynthesis and also that 2 mol of NADPH is inevitably generated at the step of GapN from 1 mol of glucose, the engineered strain can theoretically supply NADPH required for 1-lysine biosynthesis up to the yield of 0.5 mol 1-lysine/mol sugar, irrespective of the flux distribution between glycolysis and the PPP. Since the yield by strain RE2/pCAK311 (∼0.28 mol/mol) is still below the theoretical upper limit, the engineered strain is supposed to supply a sufficient amount of NADPH to its 1-lysine biosynthesis exclusively through the reconstructed glycolytic pathway, and therefore, the involvement of the PPP in NADPH supply may be insignificant in the engineered strain.

Our approach increased the 1-lysine yield by 70% on glucose, 120% on fructose, and 100% on sucrose. The higher gain on fructose than on glucose seems reasonable, because in *C. glutamicum* with GapA, the limitation of NADPH for 1-lysine production is known to become more serious when fructose is used, due to there being less flux through the PPP on fructose than on glucose (14). This means that the introduction of
GapN, which allows sufficient NADPH supply irrespective of the flux distribution between glycolysis and the PPP, can overcome such a sugar dependence problem. It should also be noted that the gains are generally higher than those obtained by the already established approach in which the supply of NADPH is increased through a redirection of carbon from glycolysis into the PPP (2, 3, 20, 25). The dilemma for the previous approach is that supplying carbon through the PPP is less advantageous than supplying it via the glycolytic pathway in terms of carbon yield because the former pathway inevitably involves the release of 1 mol of carbon dioxide accompanied by the oxidation of 1 mol of hexose. Our strategy for supplying NADPH during glycolysis can solve this dilemma and thus seems to have an advantage for the improvement of amino acid production.

Our strategy of expressing the NADP-dependent GapN from \textit{S. mutans} instead of the native NADP-dependent GAPDH can provide an additional source of NADPH during glycolysis, thus facilitating NADPH-dependent pathways. Similar strategies have resulted in increases in NADPH-dependent product synthesis in two other organisms. An increase in ethanol production from \textit{\textalpha{-}xylose} by \textit{Saccharomyces cerevisiae} has been shown by overexpression of a fungal NADP-dependent GAPDH (34). Martínez et al. (16) also showed improvement in lycopene and \textepsilon{-}caprolactone synthesis by overexpression of a \textit{Clostridium acetobutylicum} NADP-dependent GAPDH in \textit{Escherichia coli}. These previous experiments utilized NADP-dependent GAPDHs that accept both NADP and NAD, and in \textit{Escherichia coli}. These previous experiments utilized NADP-dependent GAPDHs that accept both NADP and NAD, and in


