Development of biotin prototrophic and hyper-auxotrophic
Corynebacterium glutamicum strains toward biotin production

Running title: Engineering of C. glutamicum biotin auxotrophy

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ABSTRACT To develop the infrastructure for biotin production through naturally biotin-auxotrophic Corynebacterium glutamicum, we attempted to engineer this organism into a biotin prototroph and a biotin hyper-auxotroph. To confer biotin prototrophy on this organism, the co-transcribed bioBF genes of Escherichia coli were introduced into the C. glutamicum genome, which originally lacks the bioF gene. The resulting strain still required biotin for growth but it could be replaced by exogenous pimelic acid, a source of the biotin precursor pimelate thioester linked to either CoA or acyl carrier protein (ACP). To bridge the gap between the pimelate thioester and its dedicated precursor acyl-CoA (or ACP), the bioI gene of Bacillus subtilis, which was encoding a P450 protein that cleaves a carbon-carbon bond of an acyl-ACP to generate pimeloyl-ACP, was further expressed in the engineered strain by using a plasmid system. This resulted in a biotin prototroph that is capable of the de novo synthesis of biotin. On the other hand, the bioY gene responsible for biotin uptake was disrupted in wild-type C. glutamicum. Whereas the wild strain required approximately 1 µg of biotin per liter for normal growth, the bioY disruptant ΔbioY required approximately 1 mg of biotin per liter, almost three orders of magnitude higher than the wild-type level. Strain ΔbioY showed a similar high requirement for the precursor dethiobiotin, a substrate for bioB-encoded biotin synthase. To eliminate the dependency on dethiobiotin, the bioB gene was further disrupted in both the wild strain and the ΔbioY strain. By selectively using the resulting two strains (ΔbioB and ΔbioBY) as indicator strains, we developed a practical biotin-bioassay system that can quantify biotin in the seven-digit range, from approximately 0.1 µg to 1 g per liter. This bioassay proved that the engineered biotin prototroph of C. glutamicum produced biotin directly from glucose, albeit at a marginally detectable level (approximately 0.3 µg per liter).
INTRODUCTION

Biotin, also known as vitamin B7, has a crucial function in metabolism as a cofactor in different biotin-dependent carboxylases (1, 2). In addition to its biological significance, biotin is important commercially because of its various applications in the pharmaceutical, cosmetic, food, and livestock industries. The global market for biotin is estimated to be 10 to 30 tons and several hundred million U.S. dollars per year (3, 4). Due to the lack of an efficient method of producing biotin through fermentation, its production has depended on a multi-step chemical process originally developed by Goldberg and Sternbach (4-6). However, there is an increasing interest in the development of environmentally friendly fermentation methods using renewable feedstocks to produce biotin.

From the 1980s to the early 2000s, many research groups attempted to develop biotin-producing strains from various bacterial species, including Escherichia coli, Serratia marcescens, Bacillus subtilis, Psuedomonas sp., Kurthia sp., Agrobacterium/Rhizobium, and Bacillus sphaericus (4). Although some of these attempts came close to a practical level (almost 1 g/liter per day), none of them resulted in an industrial process that would allow cost-effective production. However, today, hundreds of bacterial genome sequences have become available. In addition, the technology and strategies for molecular strain development have progressed greatly in recent years. At the same time, recent basic studies have deepened our understanding of biotin biosynthesis (7, 8). For example, the biosynthesis of the biotin pimelate moiety has long been an enigma, but intriguing models have recently been proposed for E. coli and B. subtilis (Fig. 1). In the models, the E. coli BioC-BioH pathway uses fatty acid synthetic enzymes to allow the elongation of a temporarily methylated malonate moiety
to a pimelate moiety (9). In *B. subtilis*, the pimelate moiety is generated by the oxidative
cleavage of fatty acyl chains by the P450 protein BioI (10). Based on these advances in
technologies and knowledge, it seems worthwhile to attempt biotin fermentation once
again.

We have long been working on the amino acid-producing microorganism
*Corynebacterium glutamicum*. Based on a long track record as an industrial amino acid
producer (11), this microorganism has been developed not only as a producer of amino
acids but also as a potential workhorse for the production of a wide variety of chemicals
from renewable feedstocks (12, 13). *C. glutamicum* now has an expanded product
portfolio that includes commodity chemicals (e.g., lactate, succinate,
poly-3-hydroxybutyrate, 1,2-propanediol), fuels (e.g., ethanol, isobutanol), and
heterologous proteins (e.g., transglutaminase, human epidermal growth factor). With
regard to biotin, there are some genetic and functional studies on the biotin biosynthesis
genes *bioADB* (14-17), the biotin uptake genes *bioYMN* (18), and other biotin-related
genes, such as *bioQ* (19) and *birA* (20) encoding a transcriptional regulator and biotin
protein ligase, respectively. However, there are no reports of the production of biotin
directly from sugar using *C. glutamicum*. This is likely because it is a biotin auxotroph
and no prototrophic derivative for biotin has yet been obtained. If *C. glutamicum* could
be altered to be made capable of the *de novo* synthesis of biotin, this organism might be
a promising host for the production of biotin. To examine this possibility, we started to
build an infrastructure for biotin production by *C. glutamicum*. One prerequisite for that
purpose is obviously to generate a biotin-prototrophic host strain. In addition, a simple
and efficient assay system for biotin is also an essential part of the infrastructure to
accelerate strain improvement.
Microbiological assays using biotin-auxotrophic microorganisms such as *Lactobacillus* and yeast as indicator strains have been widely used for the quantitative determination of biotin in natural materials (21, 22). However, the practicable ranges of biotin determination are so low (usually up to 1 mg/liter) that this method has the drawback of an inability to directly quantify biotin in samples containing it in high concentrations. *C. glutamicum* can also be used as an indicator strain, but it requires biotin at a very low level like other biotin auxotrophs and is only suited for quantification of very low concentrations of biotin. In this study, we found that disruption of the *bioY* gene, which is responsible for the uptake of biotin (18), dramatically enhances the biotin requirement of *C. glutamicum*. Based on these findings, we developed a practical biotin-bioassay system that allows the direct quantification of relatively high concentrations of biotin and is thus applicable to the direct screening of potent biotin producers with industrial significance.

Here we describe two important parts of the infrastructure toward biotin production by *C. glutamicum*: (i) the engineering of *C. glutamicum* into a biotin prototroph, and (ii) the development of a practical wide-range biotin-bioassay system.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The wild-type *C. glutamicum* strain ATCC 13032 was used in this study. *E. coli* K-12 W3110 and *B. subtilis* RM125 were used as donors of the genomic DNA for amplifying the biotin biosynthesis genes. *E. coli* DH5α was used as a host for DNA manipulation. Plasmid pCS299P (23), a *C. glutamicum*-*E. coli* shuttle vector, was used to clone the polymerase chain reaction (PCR) products. Plasmid pESB30 (23), which is nonreplicative in *C. glutamicum*, is a vector for gene
replacement in *C. glutamicum*. Plasmid pBBioI^gap^, for the expression of the *B. subtilis* bioI gene in *C. glutamicum*, was constructed so that the bioI gene was constitutively expressed under the promoter of the *C. glutamicum* gapA gene. For this purpose, the coding region of bioI was PCR amplified using primers bioIFusF and bioIdown90RSalI with *B. subtilis* genomic DNA as a template. On the other hand, the genomic region from -1 to -522 bp upstream of the gapA gene, which comprises its promoter, was amplified using primers PgapASalIF and bioIFusR with *C. glutamicum* genomic DNA. These two fragments were fused by PCR with primers PgapASalIF and bioIdown90RSalI. The resulting 1.8 kb fragment was digested with SalI and then ligated to SalI-digested pCS299P to yield pBBioI^gap^.

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* (BA000036), *B. subtilis* (AL009126), and *E. coli* (AP009048), which are publicly available at [http://www.genome.jp/kegg/genes.html](http://www.genome.jp/kegg/genes.html).

**Media and culture conditions.** Complete medium BY and minimal medium MM, not supplemented with biotin, were used as basal media for the growth of *C. glutamicum* strains (24). Solid plates were made by the addition of Bacto agar (Difco) to 1.6%. The agar used for MM plates was washed five times with distilled water to remove biotin and biotin-like nutrients in the agar. When required, kanamycin was added at a final concentration of 20 mg per liter. For growth test in liquid culture, 0.05 ml of the first-seed culture grown aerobically for 8 h in BY medium supplemented with 1 mg of biotin per liter was inoculated into 5 ml of MM medium and cultivated for 32 h to deplete biotin in the culture. The resulting second-seed culture was harvested, washed three times with saline, and resuspended in 5 ml of MM medium. The main culture was
started by inoculating 0.1 ml of the biotin-depleted second-seed culture into 5 ml of MM medium supplemented with indicated concentrations of biotin, dethiobiotin, or pimelic acid. All liquid cultures were performed at 30°C in L-type test tubes on a Monod shaker at 48 strokes per min. For growth of *E. coli* and *B. subtilis*, Luria-Bertani broth or agar (25) was used.

**Recombinant DNA techniques.** Standard protocols (25) were used for the extraction of *B. subtilis* and *E. coli* chromosomal DNA, for the construction, purification, and analysis of plasmid DNA, and for the transformation of *E. coli*. The extraction of *C. glutamicum* chromosomal DNA and transformation of *C. glutamicum* by electroporation were carried out as described previously (24). PCR was performed using a DNA thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA) using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). Sequencing to confirm 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 (Applied Biosystems). The subsequent electrophoresis analysis was carried out using Pageset SQC-5ALN 377 (Toyobo, Osaka, Japan).

**Strain construction.** For the chromosomal deletion of *bioY* and *bioB*, plasmids pCΔbioY and pCΔbioB, which contained the corresponding genes with internal deletions, respectively, were used to replace the wild-type chromosomal genes with the deleted genes. For the construction of plasmid pCΔbioY, the 5’ region of the *bioY* gene was PCR amplified using primers Cgl1958up600F and Cgl1958FusR with C.
*glutamicum* genomic DNA as a template. Similarly, the 3’ region of the gene was amplified using primers Cgl1958FusF and Cgl1958down600RBglII. The 5’ and 3’ regions were fused by PCR using primers Cgl1958up600F and Cgl1958down600RBglII. The resulting 1.3 kb fragment contained the deleted *bioY* gene, which was shortened from 639 to 90 bp by in-frame deletion of the inner sequence. The fragment was digested with BglII and then ligated to BamHI-digested pESB30 to yield pCΔbioY. For the construction of plasmid pCΔbioB, the 5’ and 3’ regions of the *bioB* gene were amplified using two pairs of primers: the pair comprising bioBup210BamHIF and bioBFusR3 and the pair comprising bioBFusF3 and bioBdown10BamHIR, respectively. Two fragments were fused by PCR using primers bioBup210BamHIF and bioBdown10BamHIR. The resulting 0.9 kb fragment containing the deleted *bioB* gene, which was shortened from 1005 to 705 bp by in-frame deletion of the inner sequence. This fragment was digested with BamHI and then ligated to BamHI-digested pESB30 to yield pCΔbioB. The defined chromosomal deletion of the individual gene was accomplished using each plasmid via two recombination events as described previously (26).

For the chromosomal insertion of the *E. coli* *bioBF* genes, plasmid pEbioBF was used to insert the *E. coli* genes into the nucleotide position between 1,828,311 and 1,828,312 of the *C. glutamicum* ATCC 13032 chromosome. The site is located in the center of an approximately 2.7 kb non-coding region that spans from nucleotide position 1,826,938 to 1,829,684. For the construction of plasmid pEbioBF, the region from genomic position 1,828,312 to 1,829,084 was amplified using primers ncrFFbaI and bioBFusR with *C. glutamicum* genomic DNA (for convenience, the fragment is referred to as fragment 1). Similarly, the region from nucleotide position 1,827,576 to 1,828,311
was amplified using primers bioFFusF and ncrRFbaI (fragment 2). The region
comprising bioBF genes, which are constituents of the bioBFCD operon, was amplified
using primers bioBFusF and bioFFusR with E. coli genomic DNA (fragment 3).
Fragments 1, 2, and 3 were fused by PCR in a stepwise manner. The resulting 3.8 kb
fragment was digested with BclI and then ligated to the BamHI-digested pESB30 to
yield plasmid pEbioBF. The chromosomal insertion of the E. coli bioBF genes was
accomplished using the plasmid via two recombination events as described previously
(26).

Bioassays for biotin. The bioassay plates consisted of two layers per plate: 15
ml of biotin-free MM-bottom agar (1.5%) and 3 ml of biotin-free MM-top agar (0.8%).
The MM-top agar was supplemented with 0.1 ml of indicator-cell solution that was
prepared in the same way as the biotin-depleted second-seed culture for growth test in
liquid culture, described above. The bioassay plates were loaded with sterilized paper
disks supplemented with 100 μl of different concentrations of biotin. After overnight
culture at 30ºC, the resulting halos were measured.

Agar piece assays for biotin production. The agar piece assay was carried out
basically as described previously (24). First, biotin-free MM agar plates with and
without indicated concentrations of dethiobiotin or pimelic acid were cut out with a cork
borer (6 mmΦ) to make agar pieces, followed by placing them separately in another
Petri dish. Then, wild-type ATCC 13032 and the engineered strains BF-3 and BFI-4
grown on an MM agar plate with or without 1 μg of biotin per liter were spread by
toothpicks on the top of each of the agar pieces. After cultivation for 7 days at 30ºC, the
agar pieces were transferred onto the bioassay plates prepared in the same way as
described above. After overnight culture at 30°C, the resulting halos were measured.

RESULTS

Introduction of the E. coli bioBF genes into C. glutamicum. In all known
microorganisms, biotin is synthesized from a pimelate thioester linked to either CoA or
acyl carrier protein (ACP) through four enzymatic steps, as shown in Fig. 1 (4).
Genome sequencing has revealed that the biotin auxotroph C. glutamicum lacks only the
bioF gene in the four-step pathway (27). Accordingly, our first task to confer biotin
prototrophy on this organism was to fill in the gap. For this purpose, the E. coli bioF
gene was used as a gene source. In the biotin-prototrophic E. coli, bioBFCD are
overlapping genes transcribed as one transcription unit, as shown in Fig. 2 (28). Since
their expression has been suggested to be translationally coupled, we planned to express
the bioF gene from expression signals located upstream from bioB. Thus, the
coregulated bioBF gene region was inserted into the non-coding region of the
genome of wild-type C. glutamicum ATCC 13032 (Fig. 2). One isolate was designated
strain BF-3 and characterized for its growth properties. As shown in Fig. 3, strain BF-3
still failed to grow in biotin-free MM medium. However, when supplemented with an
excess amount of pimelic acid (100 mg/liter), the strain showed significant growth
despite a prolonged lag phase. Under the same conditions, wild-type ATCC 13032
continued to show no growth. These results indicate that the heterologously expressed E.
coli bioBF genes allow C. glutamicum to synthesize biotin from exogenous pimelic acid,
Additional expression of the *B. subtilis* bioI gene. Our next task was to build a route leading to pimeloyl-CoA (or ACP) from its precursor. There are two known routes for the synthesis of the pimelate thioester (7, 8). The first is the *E. coli* bioC-bioH route and the second is the *B. subtilis* bioI route, both of which depend on fatty acid synthesis at different levels (Fig. 1). Since *C. glutamicum* lacks the known genes for the synthesis of the pimelate thioester, we attempted to engineer *C. glutamicum* BF-3 using heterologous gene(s). For this purpose, we chose the *B. subtilis* bioI gene because the encoded P450 protein BioI has been shown to be able to generate a C7 pimelate moiety *in vitro* by catalyzing the oxidative C-C bond cleavage of ACP-bound long-chain fatty acids such as oleic acid (C18:1ω-9) and palmitic acid (C16:0) (10, 29, 30), both of which represent the majority of fatty acids in the membrane lipid of *C. glutamicum* (31). If the BioI protein expressed in *C. glutamicum* cells can intercept the fatty acid synthetic intermediates C16- and C18-carbon acyl-CoA (or ACP) *in vivo*, and at the same time, if some kind of *C. glutamicum* redox system can serve as the redox partner for BioI, the biotin precursor pimeloyl-CoA (or ACP) should be generated intracellularly by the cleavage of the C7-C8 bond in the long chain acyl-CoA (or ACP). Since the *B. subtilis* bioI gene is located within the bio operon bioWAFDBI and is transcribed as one transcriptional unit (32), we cloned the coding region of the bioI gene on a multi-copy vector so as to be constitutively expressed under the promoter of the endogenous gapA gene encoding glyceraldehyde 3-phosphate dehydrogenase, and then introduced the resulting plasmid pBbioI<sup>gap</sup> into *C. glutamicum* BF-3. One of the transformants, designated strain BFI-4, was characterized for its growth properties. As shown in Fig. 3, strain BFI-4 showed significant growth in biotin-free MM medium, indicating that the engineered strain is now capable of the *de novo* synthesis of biotin, probably because
the heterologously expressed *B. subtilis* bioI gene bridged the gap between pimeloyl-CoA (or ACP) and its dedicated precursor acyl-CoA (or ACP). Although the growth rate was not completely restored to the control level obtained under the biotin-supplemented conditions, strain BFI-4 was a host strain that met the minimum requirement for biotin production.

**Disruption of the bioY gene in wild-type *C. glutamicum***. Once *C. glutamicum* was engineered into a biotin prototroph, the next challenge to be addressed was the development of a simple and efficient assay system for biotin to accelerate strain improvement. Microbiological assays using biotin auxotrophs are widely used, but they have the drawback that the practicable ranges of biotin determination are limited at very low levels (usually up to 1 mg/liter). To overcome this, we attempted to disrupt a biotin uptake system composed of three components: BioM, BioN, and BioY (Fig. 1). Since BioY has been considered to be a core transporter among the three proteins in prokaryotes (33), we constructed a bioY deletion mutant through in-frame deletion of the bioY inner sequence from wild-type *C. glutamicum* ATCC 13032. The bioY disruptant ΔbioY was compared with the wild strain for its growth responses to different concentrations of biotin in both the MM plate and the liquid culture (Fig. 4). Under both conditions, the wild strain grew well when 1 µg of biotin per liter was added to the medium. On the other hand, strain ΔbioY showed no growth at 1 µg of biotin per liter or even 10 µg per liter. When 100 µg of biotin per liter was added to the medium, growth was observed for the first time but was still impaired. For normal growth, the strain required approximately 1 mg of biotin per liter, approximately 1,000-fold more than the wild-type level. Strain ΔbioY also showed poor growth even on the complete
medium BY that was assumed to contain biotin at a concentration of tens of micrograms per liter (data not shown). These phenotypes were fully complemented by plasmid-mediated expression of the bioY gene (data not shown), showing that the disruption of the bioY gene caused the high requirement for biotin. The wild strain and strain ∆bioY could grow when biotin was replaced by the precursor dethiobiotin, and strain ∆bioY showed a similar high requirement for the precursor: namely, while the wild strain required approximately 1 µg of dethiobiotin per liter for normal growth, strain ∆bioY required approximately 1 mg per liter (data not shown). This suggests that the uptake of dethiobiotin also depends on the biotin uptake system.

Further disruption of the bioB gene. To assay for biotin only, we needed to eliminate the dependency of C. glutamicum on dethiobiotin, a substrate for bioB-encoded biotin synthase. For this purpose, we disrupted the bioB gene through in-frame deletion of the inner sequence in both the wild strain and strain ∆bioY to yield strains ∆bioB and ∆bioBY, respectively. As expected, both bioB disruptants could grow in the presence of biotin, but not in the presence of dethiobiotin (data not shown). Furthermore, we confirmed that strain ∆bioBY remained in a biotin hyper-auxotroph, like strain ∆bioY, while strain ∆bioB stayed normal biotin-auxotrophic, just like the wild strain. Thus, the resulting strains ∆bioB and ∆bioBY were considered to serve our purpose as the indicator strains for wide-range biotin bioassay.

Biotin bioassays using strains ∆bioB and ∆bioBY. To examine how high the engineered strain ∆bioBY can increase the biotin-measuring range in a microbiological
assay when used as an indicator strain, we conducted a model experiment as follows

(see Materials and Methods). The bioassay plates consisted of two layers: MM-bottom agar (1.5%) and MM-top agar (0.8%). The MM-top agar was supplemented with either of the two indicator strains, ΔbioB and ΔbioBY. The bioassay plates were loaded with paper disks containing different concentrations of biotin and incubated overnight. When strain ΔbioB was used as the indicator strain, the practicable range of biotin determination was approximately 0.1 µg to 1 mg per liter (Fig. 5A). On the other hand, the use of strain ΔbioBY permitted quantification of biotin with a range from 1 mg to 1 g per liter (Fig. 5A). Based on the correlations between the halo sizes and biotin concentrations, the biotin-bioassay system developed here can quantify biotin in the seven-digit range, from approximately 0.1 µg to 1 g per liter, by selectively using the two strains ΔbioB and ΔbioBY (Fig. 5B). This range seems to be sufficient to apply this bioassay system to strain improvement for biotin production, considering that potential industrial processes for biotin production are thought to require strains capable of producing 1 g per liter of biotin from sugar (4).

Biotin-forming ability of the biotin prototroph BFI-4. Following the model experiment, we applied this biotin-bioassay system to the evaluation of the biotin-forming potential of the engineered biotin prototroph BFI-4, using wild-type ATCC 13032 and strain BF-3 as controls (Fig. 6). For this purpose, we used agar pieces for cultivation of these strains and subsequent direct assay for biotin production (see Materials and Methods). In our agar piece assay, the three strains were cultivated on MM agar pieces supplemented with and without dethiobiotin (100 mg/liter) or pimelic acid (100 mg/liter), followed by bioassay for biotin using strain ΔbioB as the indicator.
When the three strains were cultivated on the biotin-free MM agar pieces, only the biotin prototroph BFI-4 gave a very small, but undoubted halo underneath and around the edge of the piece. Based on the halo size, the biotin concentration in the piece was calculated to be approximately 0.3 µg of biotin per liter, which was several to ten times higher than the amount synthesized by wild-type *E. coli* (3, 34). This reconfirmed that strain BFI-4 did synthesize biotin from glucose, albeit in a minute amount. When the three strains were cultivated in the presence of exogenous pimelic acid (100 mg/liter), as expected, the two strains other than the wild strain gave medium-size halos for which we estimated the biotin levels to be between 10 and 100 µg per liter. This result not only indicates that the downstream pathway after pimeloyl-CoA (or ACP) can afford additional carbon flow, but it also suggests that the supply of pimeloyl-CoA (or ACP) was limiting the *de novo* biotin biosynthesis in strain BFI-4. Under the conditions supplemented with dethiobiotin (100 mg/liter), relatively large-size halos, equivalent to 0.1 to 1 mg of biotin per liter, were obtained in all three strains, but additional supplementation of pimelic acid showed no significant positive effect on either halo size. This implies that carbon flow through the biotin-biosynthetic pathway was arrested at the last biotin synthase reaction, which was too weak to fully convert oversupplied dethiobiotin to biotin in strain BFI-4. Among the three strains, the halos of strains BF-3 and BFI-4 were somewhat larger than that of the wild strain, which is reasonable because the former two strains carried the *E. coli bioB* gene on their genomes, in addition to the native *bioB* gene.

When the same set of bioassays were conducted using the biotin-high-requiring strain ΔbioBY as the indicator strain instead of strain ΔbioB, no detectable halo was observed in any agar pieces, indicating that the biotin concentrations in the pieces were
all below the detection limit (approximately 1 mg/liter at minimum) of the indicator strain.

**DISCUSSION**

Biotin is of particular interest for *C. glutamicum* because (i) biotin auxotrophy led to its discovery as a producer of the food flavoring monosodium glutamate, (ii) biotin limitation triggers glutamate production, which is the core technology for industrial glutamate production processes, and (iii) supplementation of an excess amount of biotin is a prerequisite for the efficient production of many other amino acids, including lysine and arginine, whose biosyntheses depend on the activity of biotin-dependent pyruvate carboxylase. In this way, biotin has long been a key factor for industrial amino acid production by *C. glutamicum*. Nevertheless, as far as we know, there have been no reports of engineering biotin auxotrophy of this organism and no prototrophic derivative for biotin is known. In this study, we were able to confer the capability of the *de novo* synthesis of biotin on this organism for the first time. This raises the possibility not only of developing more economic processes for amino acid production but also of creating biotin production processes by industrially important *C. glutamicum*. So far, many bacterial species have been applied to the development of biotin-producing strains, but all of them are taxonomically biotin-prototrophic microorganisms. This study is the first to exploit the possibility that a naturally biotin-auxotrophic microorganism could be engineered into a potential biotin producer.

The *E. coli* *bioBF* genes allowed *C. glutamicum* to synthesize biotin from exogenous pimelic acid. On this point, we can raise two questions. One is how exogenous pimelic acid is incorporated into the biotin-biosynthetic pathway in *C.
glutamicum. To proceed with the process, pimelic acid needs to be activated to pimeloyl-CoA (or ACP). In B. subtilis, pimeloyl-CoA synthetase encoded by the bioW gene is known to catalyze the reaction (Fig. 1), thus allowing the organism to use pimelic acid as a precursor for biotin synthesis (32). In contrast, E. coli cannot use free pimelic acid due to the lack of the enzyme, but introduction of the B. subtilis bioW gene allows E. coli to use free pimelic acid for biotin synthesis when a large amount of pimelic acid (30 mg/l) was supplemented (32). Based on these findings, C. glutamicum is considered to have some enzyme with the pimeloyl-CoA synthetase activity. Although the genome of C. glutamicum ATCC 13032 does not have a bioW homolog, some homologous enzyme such as acyl-CoA synthetase is likely to play the role. The candidates include Cgl0105, Cgl0284, Cgl0400, Cgl1198, Cgl2296, and Cgl2872, and the possibility is under investigation. The other question is why the engineered C. glutamicum strain BF-3 required a disproportionately high amount of pimelic acid (100 mg/liter) for biotin synthesis, as is the case with E. coli (32). In relation to this, it has been suggested in E. coli and several other bacteria that there was no permease for pimelic acid and that exogenous pimelic acid was taken up into cells by passive diffusion (35). The absence of any permease for pimelic acid could be the reason for the high requirement for pimelic acid in E. coli and probably in C. glutamicum. The prolonged lag phase in the pimelic acid-supplemented culture of strain BF-3 (Fig. 3) could also be explained by the predicted uptake limitation.

The heterologously expressed B. subtilis bioI gene could allow C. glutamicum cells to supply the pimelate moiety into the biotin-biosynthetic pathway. Presumably, the pimelate moiety would be generated by the oxidative cleavage of fatty acid-biosynthetic intermediates, acyl-CoA (or ACP), that is destined for incorporation
into the membrane lipid. Fatty acid synthesis in most bacteria such as *E. coli* and *B. subtilis* is catalyzed by individual, nonaggregating enzymes (FAS-II) and the products of FAS-II are ACP derivatives (36). In contrast, the *Corynebacterianeae*, including *C. glutamicum* and closely related *Corynebacterium ammoniagenes* (previously referred to as *Brevibacterium ammoniagenes*) use eukaryotic-type multienzyme complexes (FAS-I) for fatty acid synthesis (37, 38) and the products of FAS-I have been assumed to be CoA derivatives (39). Based on these, the possible substrates for BioI in *C. glutamicum* cells are likely to be acyl-CoAs rather than acyl-ACPs, whereas the latter ACP-derivatives are thought to be the physiological substrates in *B. subtilis* (10, 30). Since BioI catalysis has not been tested with acyl-CoAs (8), the question of CoA-derivatives or ACP-derivatives remains speculative. Another possibility is that free fatty acids may be the substrates for BioI because BioI is known to utilize a range of free fatty acids as substrates in the carbon-carbon bond cleavage reaction *in vitro*, albeit less specifically (10). In this respect, we have recently found that during growth on glucose (1%), wild-type *C. glutamicum* ATCC 13032 excretes a detectable amount of free oleic acid (approximately 0.9 mg/liter) that is the major fatty acid in the *C. glutamicum* membrane lipid. This observation, coupled with the present observation that *C. glutamicum* BF-3 was able to use free pimelic acid to synthesize biotin, seems to leave open the possibility of the cleavage of free fatty acids by BioI to generate pimelic acid. If this is the case, simultaneous expression of the *B. subtilis* bioW gene encoding pimeloyl-CoA synthetase may facilitate incorporation of free pimelic acid into the biotin-biosynthetic pathway.

In this study, we demonstrated that coexpression of the *E. coli* bioBF genes along with the *B. subtilis* bioI gene could ultimately confer the capability of the *de novo*
synthesis of biotin on *C. glutamicum*. However, the carbon flow down the biotin-biosynthetic pathway seems to be the minimum level needed to support cell growth, judging from the somewhat retarded growth of strain BFI-4 on biotin-free MM medium (Fig. 3). The ameliorating effect of pimelic acid supplementation on the growth (Fig. 3) suggests that carbon flow from glucose to biotin is limited somewhere in the upstream of pimeloyl-CoA (or ACP) in the engineered strain BFI-4. The limited availability of the precursor for biotin biosynthesis is also supported by the observation that supplementation with exogenous pimelic acid resulted in biotin overproduction (Fig. 6). Considering a series of these observations, the BioI reaction could be the most plausible rate-limiting step in the biotin-biosynthetic pathway of the engineered strain. In this regard, it should be noted that BioI is a cytochrome P450 protein that requires a redox partner system(s) to shuttle electrons from NAD(P)H to the protein (10, 40). In the *B. subtilis* P450 BioI system, either or all of one ferredoxin (Fer) and two flavodoxins (YkuN, YkuP) have been suggested to be the natural redox partner(s) supporting electron transfer to BioI *in vivo* (41). In this study, introduction of the BioI protein alone enabled *C. glutamicum* cells to drive its function, suggesting that some kind of a *C. glutamicum* redox system served as a temporary redox partner for BioI. Actually, the *C. glutamicum* genome indicated the presence of a number of potential redox systems, including putative ferredoxins, flavodoxins, and related proteins (e.g., Cgl0549, Cgl1102, Cgl2959, Cgl1644, Cgl2532), and thus one or more of these proteins are assumed to mediate electron transfer to BioI in *C. glutamicum* cells. However, since none of these endogenous redox proteins seem to work like the natural partner(s) of BioI, interprotein electron transfer is likely a rate-limiting step in driving P450 BioI catalysis. If that is true, coexpression of the *bioI* gene and the gene(s) for the natural
redox partner(s) may enable efficient electron transfer to BioI, thereby improving the BioI reaction.

Along with the optimization of the BioI catalytic activity, the sufficient supply of its possible substrates acyl-CoA (or ACP) would be crucial for accelerating the BioI reaction. Increasing carbon flow into the fatty acid-biosynthetic pathway is therefore an important consideration in improving biotin production. With regard to fatty acid biosynthesis in *C. glutamicum*, its detailed regulatory mechanism is not fully understood and it is only recently that the relevant biosynthesis genes were shown to be transcriptionally regulated by the TetR-type transcriptional regulator FasR (42). To our knowledge, no attempt has been made to improve carbon flow into the pathway. Actually, there is no report of the production of fatty acids from sugar by using *C. glutamicum*. However, in the middle of this work, we found out that defined genetic modifications leading to deregulation of the fatty acid-biosynthetic pathway resulted in the production of considerable amounts of fatty acids directly from glucose in this organism (M. Ikeda and S. Takeno, unpublished data). This finding suggests that deregulation of the fatty acid-biosynthetic pathway would cause increased carbon flow down the pathway and also that the oversupplied fatty acids would be excreted into the medium without undergoing degradation in this organism. The latter hypothesis is supported by the *C. glutamicum* genome information, which shows the lack of some of the genes responsible for the β-oxidation of fatty acids (43). The fatty acids that were overproduced extracellularly in our experiment (flask cultivation with 1% glucose) included oleic acid and palmitic acid, which are major fatty acids in the *C. glutamicum* membrane. The titer of the total fatty acids and the conversion yield on glucose were approximately 300 mg/liter and 3% (w/w), respectively. Although the usefulness of the
engineered fatty acid producer as a host for biotin production remains to be evaluated, the fatty acid yield on glucose seems significant enough to achieve a practical level of biotin production. Therefore, our next task will be to examine how the carbon is channeled into the \textit{biol} route to pimeloyl-CoA (or ACP) and thence to biotin through the four-step pathway (Fig. 1).

In parallel to engineering the host for biotin production, we have developed a practical biotin-bioassay system for facilitating strain improvement. The key to this development is the finding that the disruption of \textit{bioY} enhances the biotin requirement of \textit{C. glutamicum} cells by almost three orders of magnitude. To the best of our knowledge, this study is the first to demonstrate the application of the \textit{bioY} mutant to a biotin bioassay system. With respect to biotin uptake, multiple systems are suggested to exist in prokaryotes, including the BioYMN system, which is considered to constitute tripartite transporters containing ATP-binding cassettes (33). \textit{C. glutamicum} also has \textit{bioYMN} homologs, and the predicted function of the gene products has recently been verified by transport assays with radio-labeled biotin (18). However, since attempts to disrupt the system failed (18), the phenotype of the disruptant remained unclear.

Although one could expect that the disruption of \textit{bioY} in this organism would lead to an increase in the biotin requirement, the approximately 1,000-fold increase was beyond our expectations. The BioY protein in prokaryotes is the central unit of the biotin transporter and mediates biotin uptake by itself while BioM and BioN encode an ATPase and permease, respectively, of an ABC-type transporter and are considered to be needed to convert the system into a high-affinity transporter (33). Taking this into consideration, it seems reasonable to assume that the \textit{bioY} disruption in this study would result in a complete loss of the biotin-uptake capability of the system even when the
other two components BioMN remain. This means that a further increase in the biotin requirement would not be expected by the deletion of the whole *bioYMN* gene set from the genome. On the other hand, disruption of either or both *bioMN* instead of *bioY* is likely to more or less increase the biotin requirement of the wild strain, considering their predicted roles in biotin uptake efficiency. However, since the *bioY* disruption has satisfied our purpose, those additional experiments have not yet been carried out. In this study, the *bioY* disruptant still grew under the biotin excess conditions, but this is probably due to the entry of biotin into the cells by passive diffusion, as was observed in *E. coli* (44).

So far, direct screening of potent biotin producers by microbiological assays have been hampered by their low measurable ranges of biotin concentrations. However, this study has made it possible to enhance the quantification limits of biotin concentrations to almost three orders of magnitude, which is considered adequate for the direct quantification of industrially significant levels of biotin. We believe that this assay system will assist significantly in strain development for biotin production.

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**FIGURE LEGENDS**

**FIG 1** Biotin-biosynthetic pathways and the relevant genes in *C. glutamicum*. The biotin precursor pimelate thioester is either a CoA-derivative or an acyl carrier protein (ACP)-derivative. The products of the FAS-I type fatty acid synthetases encoded by *fasA* and *fasB* are considered to be acyl-CoAs because closely related *C. ammoniagenes* (previously referred to as *Brevibacterium ammoniagenes*) has been shown to generate CoA derivatives (39). The biotin-biosynthetic pathway of *C. glutamicum* is incomplete due to the lack of the *bioF* gene and probably the gene(s) for the *de novo* synthesis of pimeloyl-CoA (or ACP). For the synthesis of a pimelate moiety, two different routes
have been proposed: the *E. coli* bioC-bioH route (broken arrows) and the *B. subtilis* bioI route (grey thick arrow). Both routes are believed to depend on fatty acid synthesis, but at different levels. In *E. coli*, BioC catalyzes methylation of malonyl-CoA to form malonyl-CoA methyl ester, which enters the fatty acid-biosynthetic pathway to generate pimeloyl-ACP methyl ester after two cycles of the chain elongation (9). The methyl ester moiety is cleaved by BioH to produce the biotin precursor pimeloyl-ACP (9). In *B. subtilis*, BioI catalyzes oxidative C-C bond cleavage of long chain acyl-ACPs to produce pimeloyl-ACP (10). The process of incorporating exogenous pimelic acid into the biotin-biosynthetic pathway remains unclear in *C. glutamicum* whereas in *B. subtilis* this step is catalyzed by the bioW gene product (32). The uptake of pimelic acid is considered to occur by passive diffusion, as is the case with *E. coli* and several other bacteria (35). In this study, the *E. coli* bioBF genes and the *B. subtilis* bioI gene were introduced into *C. glutamicum* for establishing the biotin prototroph, while the endogenous bioY gene was deleted in *C. glutamicum* for establishing the biotin hyper-auxotroph.

**FIG 2** Schematic diagram of the creation of strain *C. glutamicum* BF-3 carrying the *E. coli* bioBF genes on its genome. The *E. coli* genomic region comprising the bioBF gene cluster and its promoter/operator sequence (P/O) was cloned into a vector for gene replacement, followed by integration into the non-coding region in the *C. glutamicum* genome.

**FIG 3** Growth of wild-type strain ATCC 13032, strain BF-3, and the pBbioIgap carrier BFI-4. Cultivations were carried out in biotin-free MM medium (○) and MM medium
supplemented with 100 mg of pimelic acid (▲) or 1 μg of biotin (■) per liter. Values are means of replicated cultures, which showed <5% differences between each other.

**FIG 4** Growth responses of wild-type strain ATCC 13032 and its bioY-disrupted strain ΔbioY to biotin. (A) Appropriate dilutions (approximately 10^3 cells/ml) of cultures were spread onto MM agar plates and cultured at 30°C for 1 day under the indicated biotin concentrations. The pictures show one representative result of three independent experiments. (B) Cultivations were carried out at 30°C in MM liquid culture with 0 μg (♦), 0.1 μg (■), 1 μg (□), 10 μg (△), 100 μg (▲), and 1000 μg (●) of biotin per liter. Values are means of replicated cultures, which showed <5% differences between each other.

**FIG 5** Bioassays of different concentrations of biotin using strain ΔbioB and strain ΔbioBY as indicator strains. (A) The two indicator strains were tested for the ability to form halos on MM agar plates with paper disks supplemented with 100 μl of various concentrations of biotin. The plates were incubated overnight at 30°C. The pictures show one representative result of three independent experiments. (B) The correlations between biotin concentrations and halo sizes formed by strain ΔbioB (white column) and strain ΔbioBY (gray column) were shown. Values are means and standard deviations of three independent experiments.

**FIG 6** Biotin-forming ability of strain BFI-4 in agar piece assays. The engineered biotin prototroph BFI-4, as well as wild-type strain ATCC 13032 and strain BF-3, was cultivated on MM agar pieces with and without 100 mg of pimelic acid or dethiobiotin
per liter. After cultivation for 7 days, the agar pieces were transferred onto bioassay plates containing strain ΔbioB as the indicator strain. The plates were incubated overnight at 30°C. The pictures show one representative result of three independent experiments. Strains ATCC 13032 and BF-3, both biotin auxotrophs, appear to have grown on the biotin-free MM agar pieces with no supplementation, but this was certainly due to the carry-over of biotin.
Glucose $\rightarrow$ Acetyl-CoA

$\rightarrow$ Malonyl-CoA

TCA cycle $\rightarrow$ Fatty acid synthesis

$\rightarrow$ Acyl-CoA (ACP)

$\rightarrow$ Pimeloyl-ACP methyl ester

$\rightarrow$ Malonyl-CoA methyl ester

$\rightarrow$ Acyl-CoA (ACP)

Phospholipid $\rightarrow$ Biotin

$\rightarrow$ BioM

$\rightarrow$ BioN

$\rightarrow$ BioY

$\rightarrow$ BioH $E.\ coli$

$\rightarrow$ BioI $B.\ subtilis$

$\rightarrow$ BioW $B.\ subtilis$

Pimeloyl-CoA (ACP) $\rightarrow$ Malonyl-CoA

$\rightarrow$ (bioF) bioA bioD

$\rightarrow$ Dethiobiotin

$\rightarrow$ bioB

$\rightarrow$ Biotin

$\rightarrow$ Pimelic acid

CoA

FIG. 1. Ikeda
C. glutamicum genome

1000 kb

E. coli genome

Cgl1736
Cgl1735
Cgl1734
Cgl1733

bioB
bioF

ybhC ybhB bioA

bioB bioC bioD uvrB

bioAD

pEbioBF

bioYMN

C. glutamicum wild

Cgl1736
Cgl1735
Cgl1734 Cgl1733

C. glutamicum BF-3

bioB bioF

Cgl1736
Cgl1735
Cgl1734 Cgl1733

FIG. 2. Ikeda
FIG. 3. Ikeda

Wild Growth (OD$_{660}$) vs. Time (h)

- Wild
- BF-3
- BFI-4

Growth (OD$_{660}$)

Time (h)
(A) Biotin concentrations (µg/liter)

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(B) Growth (OD<sub>660</sub>)

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<tr>
<td>ΔbioY</td>
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FIG. 4. Ikeda
(A) Biotin concentrations (mg/liter)

<table>
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<th>1</th>
<th>10</th>
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- **ΔbioB**

- **ΔbioBY**

(B) Halo size (cm)

**FIG. 5. Ikeda**
<table>
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<th>Supplementation</th>
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**Indicator strain:** ΔbioB

FIG. 6. Ikeda