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In vivo roles of fatty acid-biosynthetic enzymes in biosynthesis of biotin and α-lipoic acid in *Corynebacterium glutamicum*

***Running title*:** Origin of biotin and lipoic acid in *C. glutamicum*

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ABSTRACT For fatty acid biosynthesis, *Corynebacterium glutamicum* uses two type I fatty acid synthases (FAS-I), FasA and FasB, in addition to acetyl-CoA carboxylase (ACC) consisting of AccBC, AccD1, and AccE. The *in vivo* roles of the enzymes in supplying precursors for biotin and α-lipoic acid remain unclear. Here, we report genetic evidence demonstrating that the biosynthesis of these cofactors is linked to fatty acid biosynthesis through the FAS-I pathway. For this study, we used wild-type *C. glutamicum* and its derived biotin-vitamer producer BFI-5, which was engineered to express *Escherichia coli bioBF* and *Bacillus subtilis bioI*. Disruption of either *fasA* or *fasB* in strain BFI-5 led to decreased production of biotin-vitamers, whereas its amplification contributed to increased production, with a larger impact of *fasA* in both cases. Double disruptions of *fasA* and *fasB* resulted in no biotin-vitamer production. The *acc* genes showed a positive effect on production when amplified simultaneously. Augmented fatty acid biosynthesis was also reflected on pimelic acid production when carbon flow was blocked at the BioF reaction. These results indicate that carbon flow down the FAS-I pathway is destined for channeling into the biotin-biosynthetic pathway, and that FasA in particular has a significant impact on precursor supply. In contrast, *fasB* disruption resulted in auxotrophy for lipoic acid or its precursor octanoic acid in both wild-type and BFI-5 strains. The phenotypes were fully complemented by plasmid-mediated expression of *fasB*, but not *fasA*. These results reveal that FasB plays a specific physiological role in lipoic acid biosynthesis in *C. glutamicum*.

IMPORTANCE For the *de novo* biosynthesis of fatty acids, *C. glutamicum* exceptionally uses eukaryotic, multifunctional type I fatty acid synthase (FAS-I) system comprising FasA and FasB, in contrast to most bacteria, such as *E. coli* and *B. subtilis*,

which use individual, nonaggregating type II fatty acid synthase (FAS-II) system. In this study, we reported genetic evidence demonstrating that the FAS-I system is the source of the biotin precursor *in vivo* in the engineered biotin-prototrophic *C. glutamicum* strain. This study also uncovered the important physiological role of FasB in lipoic acid biosynthesis. Here we present a FAS-I enzyme that functions in supplying the lipoic acid precursor, though its biosynthesis has been believed to exclusively depend on FAS-II in organisms. The findings obtained here provide new insights into the metabolic engineering of this industrially important microorganism to produce these compounds effectively.

INTRODUCTION

Biotin and α-lipoic acid are important sulphur-containing compounds with fatty acid chain-like moieties: biotin is a C, N, S-heterocyclic ring with a C-5 pentanoic fatty acid chain, and lipoic acid is a C-8 octanoic fatty acid chain with thiol groups at the C-6 and C-8 carbons. Both compounds are widespread in all three domains of life (1-4) and have crucial functions in cellular metabolism as cofactors. Biotin, also known as vitamin H, is covalently attached to a conserved lysine residue of biotin-dependent protein by biotin protein ligase, then mediating carboxylation and decarboxylation reactions (5, 6, 7). Such biotin-dependent carboxylases are known to exist in all domains of life (8). For example, naturally biotin-auxotrophic *Corynebacterium glutamicum* has two biotin-dependent enzymes, the anaplerotic enzyme pyruvate carboxylase and the fatty acid-biosynthetic enzyme acetyl-CoA carboxylase (9, 10), the biotinylation of which was shown to be specified by the biotin protein ligase gene *birA* (NCgl0679) (11). On the other hand, lipoic acid is essential for the function of several key enzymes involved

in oxidative and single carbon metabolism including pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and the glycine cleavage system (2, 3). Lipoic acid-prototrophic *C. glutamicum* has two lipoic acid-dependent enzyme complexes, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, in its central metabolism (12, 13). In addition to their biological significance, they are important commercially because of their various applications in the pharmaceutical, cosmetic, food, and livestock industries (2, 8, 14, 15). Due to the absence of an efficient method for producing either compound through fermentation, both biotin and lipoic acid are currently synthesized via costly multistep chemical processes (8, 16, 17). However, there is an increasing interest in the development of environmentally friendly fermentation methods that use renewable feedstocks for production.

We have long been working on the amino acid-producing microorganism *C. glutamicum*. Our current objective is to expand the potential of this bacterium for the production of fatty acids and their derivatives, especially the petroleum-derived, high-valued chemicals biotin and lipoic acid (18, 19). The first task toward this goal is to establish a route leading to each target metabolite from sugar, based on limited genetic and genomic information (20-24). According to this policy, we recently engineered naturally biotin-auxotrophic *C. glutamicum* into a biotin prototroph (18). The successful genetic modifications that enabled *C. glutamicum* to *de novo* synthesize biotin involve the heterologous expression of the *E. coli bioBF* genes and the *B. subtilis bioI* gene (Fig. 1). Likewise, German and Danish groups have developed biotin-prototrophic *C. glutamicum* by a similar approach with the use of *bioI* (25, 26). Since the *bioI* gene product BioI has been shown to be able to generate a C7 pimelate moiety *in vitro* by catalyzing the oxidative C-C bond cleavage of acyl carrier protein

97 (ACP)-bound long-chain fatty acids, such as oleic acid ($C_{18:1}$) and palmitic acid ($C_{16:0}$)
98 (27, 28), the biotin precursor pimeloyl-CoA (or -ACP) in the engineered strain could be
99 supplied from the fatty acid-synthetic intermediate acyl-CoA (or -ACP) that is destined
100 for incorporation into the membrane lipid (Fig. 1), though this remains speculative.
101 Thus, our next step is to obtain evidence showing that the source of the biotin precursor
102 *in vivo* is the fatty acid-biosynthetic pathway.

103 The situation is the same for lipoic acid biosynthesis in *C. glutamicum*. This
104 organism is assumed to be capable of the *de novo* synthesis of lipoic acid because of its
105 prototrophy for the cofactor. Studies on *E. coli* have established that the ACP derivative
106 of octanoic acid (C8) is the precursor of lipoic acid in the *de novo* biosynthetic pathway
107 (2, 3, 29). Biosynthesis includes the transfer of an octanoyl moiety to the lipoyl domain
108 of a lipoate-dependent apoenzyme (E2) by the LipB reaction, followed by introduction
109 of two sulphur atoms at the C-6 and C-8 positions of the octanoyl moiety by the LipA
110 reaction, resulting in protein-bound lipoic acid (Fig. 1). In addition to the *de novo*
111 pathway, *E. coli* has a salvage pathway to utilize exogenous free lipoic acid and
112 octanoic acid through transferring them to the lipoyl domain of the E2 subunit by the
113 lipoate-protein ligase (LplA) reaction (3, 29). As the putative *lipA* (NCgl2128) and *lipB*
114 (NCgl2127) genes are present on the *C. glutamicum* genome to form a cluster with *aceF*
115 (NCgl2126) encoding the E2 subunit (13), *C. glutamicum* is thought to *de novo*
116 synthesize lipoic acid through an octanoic acid derivative in a manner similar to that of
117 *E. coli*. The putative *lplA* gene (NCgl1029) also exists on the genome, suggesting that *C.*
118 *glutamicum* can utilize exogenous free lipoic acid and octanoic acid, like *E. coli*.
119 However, in the *de novo* synthesis of lipoic acid, the origin of the octanoyl moiety
120 remains an enigma (Fig. 1), because the *de novo* synthesis of medium-chain (8- to

10-carbon) fatty acids has not been observed in *C. glutamicum*. In this regard, it should be noted that *C. glutamicum* originally lacks the β-oxidation pathway involving fatty acid degradation (19, 30), which makes it unlikely that the medium-chain fatty acids are generated in the middle of the degradation cycles of long-chain fatty acids.

For the *de novo* biosynthesis of fatty acids from acetyl-CoA, the *Corynebacterianeae*, including *C. glutamicum*, *Corynebacterium ammoniagenes*, and *Mycobacterium tuberculosis*, exceptionally use type I fatty acid synthase (FAS-I) (31-34**)**, a eukaryotic-type multienzyme that performs successive cycles of fatty acid synthesis, into which all activities required for fatty acid elongation are integrated (35). The products of the FAS-I pathway are believed to be CoA-bound long-chain fatty acids, such as oleic acid and palmitic acid (34, 35), both of which represent the majority of fatty acids in the membrane lipid of *C. glutamicum* (36). In contrast, 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 the FAS-II pathway are ACP derivatives (37). In *E. coli*, the FAS-II pathway is thought to be the source of the C-8 octanoyl moiety required for lipoic acid synthesis (38). More specifically, octanoyl-ACP is believed to be a preferred product of β-ketoacyl-ACP synthase III *in vivo* (39).

Eukaryotic organisms, including yeast, fungi, plants, and animals, possess the FAS-I pathway in the cytoplasm (40). Recent studies have revealed, however, that they also have the bacterial type FAS-II pathway in mitochondria (41), thus raising the puzzling question of why the FAS-II pathway has been maintained in mitochondria (in addition to the cytosolic FAS-I pathway). On this point, a significant amount of data supports the idea that the mitochondrial FAS-II pathway is involved in the *de novo* synthesis of the octanoyl moiety required for lipoic acid formation in eukaryotic cells

(41-43). This hypothesis seems reasonable because all of the known lipoic acid-dependent enzymes in eukaryotes are located in mitochondria (44, 45). The known FAS systems in different organisms are summarized in Table 1.

Unlike eukaryotic organisms, *C. glutamicum* possesses the FAS-I pathway, but not the FAS-II pathway (33). How then does this bacterium generate the octanoyl moiety required for lipoic acid synthesis? Our hypothesis is that the FAS-I pathway carried by *C. glutamicum* is exceptionally responsible for the formation of the octanoyl moiety. On this point, it is worth noting that the FAS-I pathway of *C. glutamicum* consists of two type I fatty acid synthases, FasA and FasB, encoded by *fasA* and *fasB*, respectively (31, 32), just as in the case of the closely related species *C. ammoniagenes* (52). The expression of f*asA* is known to be much higher than that of *fasB*: the *fasA* transcript accounts for approximately 70% of the sum of both *fasA* and *fasB* transcripts in *C. glutamicum* cells grown on glucose (31, 52). The major FasA enzyme is essential for growth as it synthesizes membrane lipids consisting mainly of oleic acid and palmitic acid, and its deficiency is known to cause oleic acid auxotrophy (31, 52, 53). The minor FasB enzyme is thought to primarily synthesize palmitic acid, but not oleic acid, and is dispensable for growth (31, 52, 53). In this study, however, we obtained genetic evidence indicating that FasB specifically functions in supplying the octanoyl precursor of lipoic acid, and FasA and FasB thus have different physiological roles in cell growth.

Here, we describe the *in vivo* roles of the fatty acid-biosynthetic enzymes in supplying the precursors for the biosynthesis of biotin and lipoic acid using wild-type *C. glutamicum* and its derived biotin-vitamer producer. Our report shows the direct relationship between the biosynthesis of these cofactors and fatty acid biosynthesis

through the FAS-I pathway.

RESULTS

Generation of a *C. glutamicum* strain with *E. coli bioBF* and *B. subtilis bioI* on its genome. *C. glutamicum* is a natural biotin auxotroph due to the lack of the *bioF* gene and the gene(s) for the *de novo* synthesis of pimeloyl-CoA (or -ACP) (Fig. 1). We have recently constructed the *C. glutamicum* strain BF-3 which expresses the cotranscribed *E. coli bioBF* genes on its genome (18). By using this strain as a host, we demonstrated that further expression of the *B. subtilis bioI* gene by using a plasmid system resulted in a biotin prototroph, BFI-4, that is capable of the *de novo* synthesis of biotin (18). In this study, to facilitate the following strain engineering, we inserted the *bioI* gene into the non-coding regions of the genome of strain BF-3 to generate strain BFI-5 (Fig. 2). This strain showed both biotin prototrophy and the ability to produce approximately 5 μg per liter of biotin-vitamers when cultivated in MM medium (1% glucose). If biotin biosynthesis in strain BFI-5 is linked to fatty acid biosynthesis, disruption of the fatty acid biosynthesis gene(s) should affect the biotin-synthetic ability of the strain.

Effect of *fas* disruption on biotin-vitamer production. Oleic acid and palmitic acid comprise the bulk of the fatty acids found in *C. glutamicum* membrane lipids, and two functional FAS-I proteins FasA and FasB (Fig. 1) are considered to play significant roles in controlling the chain length and amounts of these fatty acids (31). To examine the effect of deficiency of either or both of them on biotin-vitamer production by strain BFI-5, we constructed *fasA*- and *fasB*-disrupted strains, designated strain BFIΔ*fasA* and strain BFIΔ*fasB*, respectively, and their double disruptant, designated strain BFIΔ*fasAB*,

193 from strain BFI-5. The performance of these three mutant strains was compared with
194 that of the parental strain BFI-5 using MM medium (1% glucose) supplemented with 10
195 μg of lipoic acid per liter in 300-ml baffled Erlenmeyer flasks. Since *fasA* disruption
196 caused the requirement of oleic acid for growth, we evaluated the oleic acid auxotrophic
197 strains BFIΔ*fasA* and BFIΔ*fasAB* under the conditions supplemented with the oleic acid
198 surfactant Tween 80. Under these conditions, the oleic acid-auxotrophic strains
199 BFIΔ*fasA* and BFIΔ*fasAB* exhibited retarded growth, probably because of the
200 inefficient utilization of Tween 80 as the source of oleoyl-CoA (or -ACP), but ultimately
201 led to almost the same growth levels as the control strain (Fig. 1S, Fig. 3A). After
202 glucose was consumed, the culture supernatant was subjected to biotin-vitamer bioassay.
203 First of all, we confirmed that supplementation of Tween 80, namely, exogenous oleic
204 acid, had little influence on biotin-vitamer production in both the control strain BFI-5
205 and strain BFIΔ*fasB* (Fig. 3A). Under such Tween 80-supplemented conditions, strains
206 BFIΔ*fasA* and BFIΔ*fasB* showed approximately 80% and 42% decreased yields of
207 biotin-vitamers, respectively, compared to the control strain. Strain BFIΔ*fasAB*
208 produced no detectable biotin-vitamer (Fig. 3A). These data suggest that both FasA and
209 FasB play significant roles in supplying carbon into the biotin-biosynthetic pathway,
210 and therefore, *fas* disruption would cause a shortage of the biotin precursor
211 pimeloyl-CoA (or -ACP). In fact, exogenous pimelic acid was shown to improve
212 biotin-vitamer production by the *fas*-disrupted strains to the levels comparable to that of
213 the control strain BFI-5: when cultivated under the conditions of supplementation with
214 pimelic acid (10 mg liter^{-1}), strains BFIΔ*fasA*, BFIΔ*fasB*, and BFIΔ*fasAB*, as well as the
215 control strain BFI-5, produced approximately 30 μg per liter of biotin-vitamers.
216

Effect of amplified fatty acid biosynthesis genes on biotin-vitamer production. Based on the above-mentioned results, it could be reasonably expected that the increased carbon flow down the fatty acid-biosynthetic pathway results in increased production of biotin-vitamers in strain BFI-5. To examine this possibility, we constructed pCaccBC and pCaccD1, high copy plasmids containing the *accBC* and *accD1*genes, respectively, under the strong promoter of the *C. glutamicum gapA* gene (Fig. S2). In addition, we constructed pCaccBCDE which carries all subunit genes for the ACC complex under the *gapA* promoter (Fig. S2). On the other hand, the intact *fasA* and *fasB* genes, both of which are approximately 9-kb long, were individually cloned into a high copy vector to generate pCfasA and pCfasB, respectively (Fig. S2). Each plasmid was introduced into strain BFI-5 and the resulting plasmid carriers were compared with the control vector carrier for biotin-vitamer production when cultivated in MM medium (1% glucose). As shown in Fig. 3B, plasmids pCaccBC and pCaccD1, which overexpress one subunit of the ACC complex, had only marginal effects on biotin-vitamer production, but plasmid pCaccBCDE, which overexpresses all subunits simultaneously, enhanced the titer by 2.7-fold. Furthermore, plasmids pCfasA and pCfasB brought about increased production by 3.5-fold and 1.6-fold, respectively. These data show that carbon through the biotin-biosynthetic pathway originates from the fatty acid-biosynthetic pathway in strain BFI-5, and that ACC and FasA have major impacts on precursor supply for biotin biosynthesis.

Effect of amplified fatty acid biosynthesis genes on pimelic acid production. Since pimeloyl-CoA (or -ACP) is the precursor of biotin-vitamers, the carbon influx into the biotin-biosynthetic pathway might be more directly reflected on pimelic acid

accumulation if the BioF reaction is blocked. To confirm this hypothesis, we constructed *C. glutamicum* WTI-1 which expresses only the *B. subtilis bioI* gene on the wild-type genome. Since the engineered strain originally lacks the *bioF* gene, carbon flow through the BioI reaction would be arrested at the BioF reaction, thereby causing accumulation of pimeloyl-CoA (or -ACP) and subsequent excretion of free pimelic acid into the medium. In fact, LC-MS/MS analysis revealed that when cultivated in fermentation medium LFG1 containing 5% glucose in 300-ml baffled Erlenmeyer flasks, strain WTI-1 and its vector carrier accumulated the expected amounts of pimelic acid (approximately 20 μg per liter) in the culture supernatants whereas wild-type ATCC 13032 did not. Following this, we introduced plasmids pCaccBCDE, pCfasA, and pCfasB into strain WTI-1, and the resulting plasmid carriers were compared with the control vector carrier for pimelic acid production under the same culture conditions. As expected, all the three plasmids, pCaccBCDE, pCfasA, and pCfasB, brought about increased production by 2.3-fold, 3.0-fold, and 1.8-fold, respectively (Table 2). These results reinforce our conclusion that the fatty acid-biosynthetic pathway is the source of the biotin precursor *in vivo* in our *C. glutamicum* strains.

Effect of *fas* disruption on lipoic acid biosynthesis. Although *fasA* deficiency causes oleic acid auxotrophy in *C. glutamicum*, it has been reported that *fasB* deficiency does not bring about any detectable auxotrophic phenotype (31). However, since *C. glutamicum* does not require exogenous lipoic acid for aerobic growth on glucose, the octanoyl moiety required for lipoic acid synthesis needs to be supplied by a specific endogenous biosynthetic route. This prompted us to examine the phenotypes of deficiency in *fasA* or *fasB* under the wild-type background. For this purpose, *fasA*- and

265 *fasB*-disrupted strains were derived from *C. glutamicum* wild-type ATCC 13032 to
266 generate strains WTΔ*fasA* and WTΔ*fasB*, respectively. These disruptants, as well as the
267 wild-type strain, were examined for their growth on agar plates under the conditions in
268 the presence and absence of oleate, lipoic acid, and octanoic acid. Fig. 4A shows the
269 results when appropriate dilutions of the cultures (approximately 10^3 cells) were spread
270 onto the plates. Strain WTΔ*fasA* showed an expected phenotype of oleic acid
271 auxotrophy. In contrast, strain WTΔ*fasB* exhibited lipoic acid-dependent growth. The
272 requirement of lipoic acid in the *fasB* mutant could be replaced by octanoic acid. For
273 normal growth on agar plates, the *fasB* mutant required a very small amount of lipoic
274 acid (0.01 μg liter^{-1}) or a disproportionately large amount of octanoic acid (1 mg liter^{-1})
275 (data not shown). The large requirement of octanoic acid compared to lipoic acid could
276 be due to the limited incorporation into the lipoic acid-biosynthetic pathway, because
277 exogenous octanoic acid needs to be taken up into cells and then activated to
278 octanoyl-CoA (or -ACP) by an enzyme with octanoyl-CoA (or -ACP) synthetase
279 activity or transferred to the apoprotein (E2) by a putative LplA enzyme. It should be
280 noted that the lipoic acid-auxotrophic phenotype of strain WTΔ*fasB* was not observed
281 on agar plates when a higher concentration of cells (approximately 10^5 cells and more)
282 was spread on the plates (Fig. S3).
283 The growth properties were further examined in liquid cultures. Unfortunately,
284 under normal conditions with 1% inoculum from the seed culture to the main culture,
285 we failed to observe lipoic acid auxotrophy for the *fasB* mutant although the *fasA*
286 mutant exhibited oleic acid auxotrophy. However, subsequent investigations revealed
287 that by decreasing the inoculum size to 0.01% or below, real phenotypes of the *fasB*
288 mutant became evident. As shown in Fig. 4B, which was conducted under the

conditions with 0.01% inoculum, the phenotypes of the *fasB* mutant observed on agar plates, namely, the auxotrophy for lipoic acid or octanoic acid, were clearly reproduced in liquid cultures. We also reconfirmed that, in the case of the *fasA* mutant, lipoic acid and octanoic acid have no stimulating effect on growth in the presence of oleic acid.

Complementation of lipoic acid auxotrophy with cloned *fasA* and *fasB*. To confirm that the lipoic acid auxotrophy of the *fasB* mutant is actually due to the loss of the FasB function, we examined the effects of plasmid-mediated expression of *fasA* and *fasB* on the phenotypes in strain WTΔ*fasB*. Introduction of pCfasB into strain WTΔ*fasB* resulted in complete recovery of growth even in the absence of lipoic acid and octanoic acid whereas pCfasA failed to restore the growth (Fig. 5). A series of these results were reproduced under the background of BFI-5 (data not shown). Thus we concluded that the FasB pathway is the source of the octanoyl moiety required for lipoic acid synthesis under our conditions, and that the FasA pathway is unable to produce sufficient amounts of the octanoyl precursor to fulfill the need for cellular lipoic acid synthesis.

DISCUSSION

From the 1980s to the early 2000s, a number of biotin-producing strains were developed from various bacterial species, including *E. coli*, *Serratia marcescens*, *B. subtilis*, *and Bacillus sphaericus* (8). Since the origin of the biotin precursor pimeloyl-CoA (or -ACP) had long been an enigma, previous attempts at strain improvement had focused on the biotin-biosynthetic pathway beginning at pimeloyl-CoA (or -ACP) (8). It should be also noted that some of those studies used feeding of relatively costly pimelic acid during fermentation to obtain high production yields (54-56). Only recently,

pimeloyl-CoA (or -ACP) synthesis seemed likely to involve fatty acid biosynthesis (2, 7). To the best of our knowledge, however, there have been no reports of increased biotin production by rationally modifying the fatty acid-biosynthetic pathway. In this study, we used the engineered biotin-vitamer producer *C. glutamicum* BFI-5 to show that carbon flow down the fatty acid-biosynthetic pathway is crucial for the biosynthesis of biotin-vitamer. Furthermore, we demonstrated that augmented fatty acid biosynthesis led to increased production of biotin-vitamers, thus concluding that the biotin precursor pimeloyl-CoA (or -ACP) originates from the fatty acid-biosynthetic pathway in strain BFI-5. In relation to this, Manandhar and Cronan have very recently reported that in *B. subtilis*, the precursor for biotin biosynthesis is free pimelic acid originated from fatty acid biosynthesis, because *bioW* encoding pimeloyl-CoA synthetase was essential for biotin biosynthesis whereas *bioI* was dispensable (57). Although the mechanism of formation of free pimelic acid without the BioI function remains to be determined, their hypothesis is that pimeloyl-ACP is directly generated by the FAS II pathway of *B. subtilis*, and then subject to thioesterase-catalyzed cleavage to generate free pimelic acid, followed by activation to pimeloyl-CoA by BioW. This hypothesis seems reasonable because pimeloyl-CoA but not pimeloyl-ACP is thought to be the substrate of *B. subtilis* BioF (57). Nevertheless, this seems not to be the case with our *C. glutamicum* strain because *C. glutamicum* BFI-5 exclusively depends on BioI for the *de novo* biotin biosynthesis. Considering that the products of *C. glutamicum* FAS-I have been assumed to be CoA derivatives (34), acyl-CoAs rather than acyl-ACPs are likely to be the substrates for BioI in the *C. glutamicum* strain, whereas the ACP derivatives are thought to be the physiological substrates in *B. subtilis* (28, 58). Taking these into consideration, it seems reasonable to assume that long-chain fatty acyl-CoAs are subject to

BioI-catalyzed oxidative cleavage to directly generate the biotin precursor pimeloyl-CoA in our *C. glutamicum* strain (Fig. 1).

Both *fasA* and *fasB* contributed to biotin-vitamer production when amplified in the engineered strain BFI-5. This could be explained as a result of the increased availability of acyl-CoAs (or -ACPs) for the BioI reaction. A larger impact of *fasA* on production than *fasB* is reasonable, considering that the expression of *fasA* is much higher than that of *fasB* (31). On the other hand, the double disruption of *fasA* and *fasB* resulted in the loss of the capability of biotin-vitamer synthesis. This means that the FAS-I pathway comprising FasA and FasB is the sole source of the biotin precursor in strain BFI-5. In this regard, it should be noted that the production experiments were carried out under conditions of supplementation with Tween 80. This is not only because *fasA* disruption caused oleic acid auxotrophy, but also because exogenous oleic acid never affected biotin-vitamer production in strain BFI-5 (Fig. 3A). The latter reason raises the question of why exogenous oleic acid failed to contribute to biotin-vitamer production. At present, it remains unclear, but it seems reasonable to speculate as follows, based on the predicted regulatory mechanism of fatty acid biosynthesis in this organism (19, 59). Under the wild-type background with respect to the *de novo* fatty acid biosynthesis, exogenous oleic acid would be taken up into cells and then activated to oleoyl-CoA (or -ACP), which would negatively regulate the *de novo* fatty acid biosynthesis so as to maintain the intracellular pool of oleoyl-CoA (or -ACP) at the steady state. In contrast, against the background of deficiency in the *de novo* fatty acid biosynthesis, the process of incorporation of exogenous oleic acid into oleoyl-CoA (or -ACP), namely, uptake of exogenous oleic acid or its activation to oleoyl-CoA (or -ACP), seems to be rate-limiting for synthesis of the membrane lipid,

361 judging from the significantly retarded growth of strains BFIΔ*fasA* and BFIΔ*fasAB*
362 compared with the parental strain BFI-5 under the conditions of supplementation with
363 Tween 80 (Fig.S1). If so, it is likely that oleoyl-CoA (or -ACP) generated through the
364 salvage route would be preferentially incorporated into the membrane lipid instead of
365 being the substrate for the BioI reaction.
366 Blocking the fatty acid-biosynthetic pathway through double disruption of *fasA*
367 and *fasB* should make strain BFI-5incapable of *de novo* biotin synthesis. Nevertheless,
368 the engineered strain did not show biotin auxotrophy on glucose. This is certainly
369 because biotin is unnecessary for growth on glucose as long as the essential fatty acid
370 oleic acid (or Tween 80) is added to the medium. In contrast, in the case of lipoic acid, a
371 genetic approach is feasible to verify the link between the fatty acid-biosynthetic
372 pathway and the source of the lipoic acid precursor, because the cofactor is
373 indispensable for *C. glutamicum* to grow aerobically on glucose as the sole carbon
374 source. In this study, we showed that disruption of *fasB* caused auxotrophy for lipoic
375 acid or octanoic acid in the wild-type strain ATCC 13032, and that the phenotypes were
376 fully complemented by plasmid-mediated expression of *fasB*. These results have proved
377 that the octanoyl moiety of lipoic acid is supplied by FasB in this organism. In this
378 respect, there are two possible mechanisms for the supply of the octanoyl moiety by
379 FasB. The first is that the octanoyl moiety is a direct product of FasB, and the second is
380 that it is derived from the FasB end product palmitic acid or its derivative by an
381 unidentified enzyme with the oxidative C-C bond cleavage activity toward long-chain
382 fatty acids. However, since the long-chain fatty acids made by FasB are also generated
383 by FasA, the second possibility seems unlikely. In fact, the requirement of lipoic acid in
384 the *fasB* mutant could not be replaced by long-chain oleic acid and palmitic acid, the

main products of FasA and FasB, respectively, on MM agar plates (data not shown). Taken together, we conclude that the octanoyl precursor of lipoic acid is a direct product of FasB in this organism, at least, under the conditions employed. The octanoyl precursor is likely to be the CoA derivative rather than the ACP derivative (Fig. 1), considering that the products of FasB have been assumed to be CoA derivatives (34). However, this remains speculative because the *C. glutamicum* LipB catalysis has not been tested with the CoA derivative. The fact that deficiency of FasA and FasB caused auxotrophy for oleic acid (31, 52) and lipoic acid, respectively, on glucose indicates that the two enzymes have basically different physiological roles.

It is noteworthy that the *C. glutamicum* FasB, a multifunctional FAS-I enzyme, can function to supply the octanoyl precursor of lipoic acid, since its biosynthesis has been believed to exclusively depend on FAS- II in organisms (29), as mentioned in the introduction. However, this raises the question of what allows FasB to generate the dedicated product octanoyl-CoA. In this respect, the FAS-I of the closely related species *C. ammoniagenes* has been reported to carry out the transacylation of long-chain fatty acids from the enzyme to CoA using its integral palmitoyl transferase activity, and to produce long-chain acyl-CoAs including palmitoyl-, oleoyl-, and stearoyl-CoA (34, 35). Based on this information, one possibility for the synthesis of octanoyl-CoA by the *C. glutamicum* FasB is that the transacylase in the FasB multienzyme complex may possess the activity of the transfer of the octanoyl moiety to CoA, even at a marginal level. In general, the chain length of its products is considered to be an inherent property of every fatty acid synthase, although the determinants remain elusive (35). Analyses of the structure-activity correlation between FasA and FasB may help to answer the question.

One of the goals of metabolic engineering for product formation is to direct as much carbon as possible from sugar into a desired product. For this goal, the supply of precursors for the relevant terminal biosynthetic pathways is of key importance for successful metabolic engineering. In this study, we demonstrated that the FAS-I pathway is the source of the precursors for both biotin and lipoic acid in *C. glutamicum*. Furthermore, this study uncovered the important physiological roles of two FAS-I enzymes, FasA and FasB, in the biosynthesis of each cofactor. The findings obtained here provide new insights into the metabolic engineering of this industrially important microorganism to produce these compounds effectively.

MATERIALS AND METHODS

Bacterial strains. The biotin-auxotrophic wild-type *C. glutamicum* strain ATCC 13032 was used in this study. *C. glutamicum* Δ*ppc* was used as an indicator strain for biotin-vitamer bioassays especially under the conditions supplemented with oleic acid. This indicator strain was derived from ATCC 13032 through disruption of the *ppc* gene encoding phosphoenolpyruvate carboxylase, one of two anaplerotic enzymes carried by this organism. The wild-type ATCC 13032, an auxotroph for biotin-vitamers, never show the biotin-vitamer auxotrophy in the presence of oleic acid. In contrast, the indicator strain Δ*ppc* remains auxotrophic for biotin-vitamers irrespective of the presence or absence of oleic acid, because *ppc* deficiency makes cells dependent on the alternative anaplerotic biotin enzyme pyruvate carboxylase which requires biotin for its activity (60). Since strain Δ*ppc*, like the wild-type ATCC 13032, is capable of synthesizing biotin from any of the biotin-vitamers, the strain can be used for bioassay for the total biotin-vitamers. *E. coli* K-12 W3110 (61) and *B. subtilis* RM125 (62) were

used as donors of genomic DNA to amplify the biotin biosynthesis genes. *E. coli* DH5α (63) was used as a host for DNA manipulation.

Plasmids. Plasmid pCS299P (64), a *C. glutamicum-E. coli* shuttle vector, was used to clone the polymerase chain reaction (PCR) products. Plasmid pESB30 (64), which is nonreplicative in *C. glutamicum*, is a vector for gene replacement in *C. glutamicum*. Plasmids pCaccBC and pCaccD1 were constructed so that *accBC* (Cgl0700, NCgl0670) and *accD1* (Cgl0708, NCgl0678) were constitutively expressed under the promoter of the endogenous *gapA* gene (Fig. S2). For the construction of pCaccBC, the coding region of *accBC* was amplified using primers accBCPgapAFusF and accBCdown100RKpnI with wild-type ATCC 13032 genomic DNA as a template. On the other hand, the genomic region comprising the *gapA* promoter was amplified using PgapAKpBgF and accBCPgapAFusR. These two fragments were fused by PCR, digested with KpnI, and then ligated to KpnI-digested pCS299P to yield pCaccBC. Similarly, for the construction of pCaccD1, the coding region of *accD1* was amplified using primers accD1PgapAFusF and accD1down150RKpnI, and the genomic region comprising the *gapA* promoter was amplified using primers PgapAKpBgF and accD1PgapAFusR. These fragments were fused by PCR, digested with KpnI, and then ligated to Kpn-digested pCS299P to yield pCaccD1. Plasmid pCaccBCDE for the constitutive co-expression of modified *accBC* where a start codon was changed to ATG, *accD1*, and *accE* (Cgl0706, NCgl0676) under the *gapA* promoter was constructed as follows (Fig. S2). The genomic regions comprising *accBC* and *accE* were separately amplified using the primer pairs of InFu-accBCf and InFu-accBCr and of InFu-accEf and InFu-accEr, respectively. On the other hand, pCaccD1 was linearized by inverse

457 PCR using primers InFu-accD1f and InFu-accD1r. The amplified genomic regions and
458 linearized pCaccD1 were fused using the In-Fusion HD Cloning Kit (Clontech
459 Laboratories Inc., Mountain View, CA) to yield pCaccBCDE. On this plasmid, the
460 *accD1*, *accBC*, and *accE* genes were tandemly arranged in this order from the *gapA*
461 promoter. The regions between the *accD1* and *accBC* ORFs and between the *accBC* and
462 *accE* ORFs were identical to the nucleotide sequences from -1 to -30 bp upstream of the
463 *accBC* gene and from -1 to -23 bp upstream of the *accE* gene, respectively.
464 Plasmid pCfasA and pCfasB, which contain the intact *fasA* gene (Cgl0836,
465 NCgl0802) and the intact *fasB* gene (Cgl2495, NCgl2409), respectively, were
466 constructed as follows (Fig. S2). For pCfasA, the genomic region comprising *fasA* and
467 its native promoter (from -1 to -332 bp upstream of *fasA*) was amplified using primers
468 fasAexpFupEcoRV and fasAexpRdownNheI. On the other hand, pCS299P was
469 linearized by inverse PCR using primers InVer-pCS299PfasAf and InVer-pCS299PfasAr.
470 The amplified genomic region and linear pCS299P were fused using the In-Fusion HD
471 Cloning Kit to yield pCfasA. For plasmid pCfasB, the genomic region comprising *fasB*
472 and its native promoter (from -1 to -617 bp upstream of *fasB*) was amplified using
473 primers fasBexpFupBlnI and fasBexpRdownNheI. On the other hand, pCS299P was
474 linearized by inverse PCR using primers InVer-pCS299PfasBf and InVer-pCS299PfasBr.
475 The amplified genomic region and linear pCS299P were fused to yield pCfasB.
476 The sequences of the primers used in this study are listed in Table 3. All
477 primers were designed based on the genomic sequences of *C. glutamicum* (BA000036),
478 *B. subtilis* (AL009126), and *E. coli* (AP009048), which are publicly available at
479 http://www.genome.jp/kegg/genes.html.
480

Media. Complete media BY and BYG (BY medium containing 1% glucose), and minimal medium MM, not supplemented with biotin, were used as basal media for the growth of *C. glutamicum* strains (65). Fermentation medium LFG1 containing 5% glucose was used for pimelic acid production (66). Solid plates were made by the addition of Bacto agar (Difco) to 1.5%. For the preparation of MM medium containing sodium oleic acid, sodium oleic acid was separately autoclaved, and then mixed with a magnesium sulfate solution and a solution containing other components to prevent insolubilization of the fatty acid. When required, the agar used for MM plates was washed five times with distilled water to remove unnecessary nutrients in the agar. For cultivation of plasmid carriers, kanamycin was added at a final concentration of 10 mg per liter. For growth of *E. coli* and *B. subtilis*, Luria-Bertani broth or agar (63) was used.

Recombinant DNA techniques. Standard protocols (63) 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 (65). 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).

505

506 **Strain construction.** For the chromosomal deletion of *fasA* and *fasB*, plasmids
507 pCΔfasA and pCΔfasB, which contained the corresponding genes with internal
508 deletions, respectively, were used to replace the wild-type chromosomal genes with the
509 deleted genes (Fig. S2). For the construction of pCΔfasA, the 5' and 3' regions of *fasA*
510 were amplified using the primer pairs of CglfasIA5'BglIIF and CglfasIAFusR and of
511 CglfasIAFusF and CglfasIA3' BglIIR, respectively. These two fragments were fused by
512 PCR, digested with BglII, and then ligated to BamHI-digested pESB30 to yield
513 pCΔfasA. Plasmid pCΔfasA carried the in-frame deleted *fasA* gene, which was
514 shortened from 8910 to 2181 bp and thus devoid of a motif sequence for the
515 3-ketoacyl-ACP synthase active site (PROSITE motif PS00606). Similarly, for the
516 construction of pCΔfasB, the 5' and 3' regions of the *fasB* gene were amplified using the
517 primer pairs of fasBupF and fasBFusR and of fasBFusF and fasBdnR, respectively.
518 These fragments were fused by PCR, digested with BclI, and then ligated to
519 BamHI-digested pESB30 to yield pCΔfasB. Plasmid pCΔfasB carried the in-frame
520 deleted *fasB* gene, which was shortened from 8991 to 93 bp. The defined chromosomal
521 deletion of the individual gene in both wild-type and BFI-5 strains was accomplished
522 using each plasmid via two recombination events as described previously (67).
523 For the chromosomal insertion of the *B. subtilis bioI* gene so as to be
524 constitutively expressed under the promoter of the endogenous *gapA* gene, plasmid
525 pBbioI^{PgapA} was used to insert the *B. subtilis* gene with the *gapA* promoter into the
526 nucleotide position between 1,827,653 and 1,827,654 of the genomes of *C. glutamicum*
527 BF-3 and wild-type ATCC 13032 to generate strains BFI-5 (Fig. 2) and WTI-1,
528 respectively. For the construction of pBbioI^{PgapA}, the region from genomic positions

1,827,654 to 1,828,204 of the ATCC 13032 genome was amplified using primers ncrFBam2 and PgapAFusR (Fragment a). Similarly, the region comprising the *gapA* promoter was amplified using PgapAFusF and bioIFusR (Fragment b). On the other hand, the region comprising the *B. subtilis bioI* gene was amplified using primers bioIFusF and bioIfusR2 (Fragment c). Moreover, the region from 1,826,948 to 1,827,653 of the ATCC 13032 genome was amplified using primers ncrFusF and ncrRBam2 (Fragment d). Fragments a, b, c, and d were fused by PCR in a stepwise manner, digested with BamHI, and ligated to BamHI-digested pESB30 to yield pBbioI^{PgapA}.

Biotin-vitamer production. A 3 ml sample of the seed culture grown in BYG medium to the mid-exponential phase at 30ºC was harvested, washed with saline to remove biotin-vitamer, and inoculated into a 300-ml baffled Erlenmeyer flask containing 30 ml of biotin-free MM medium supplemented with10 μg of lipoic acid per liter, followed by cultivation at 30ºC using a rotary shaker at 200 rpm. For the cultures of the oleic acid-auxotrophic strains BFIΔ*fasA* and BFIΔ*fasAB*, Tween 80 was added into both BYG and MM media at a final concentration of 1 g per liter. After glucose was consumed, the culture supernatant was prepared by removing cells through centrifugation and subsequent filtration with a Millex-MA filtrate unit (0.45 μm pore size; Millipore Corporation, Billerica, MA). The resulting solution was subject to bioassay of biotin-vitamers.

Bioassays for biotin-vitamer. Biotin-vitamers include not only biotin itself but the intermediates in the biotin-biosynthetic pathway, that is, the BioF product

553 7-keto-8-aminopelargonic acid (KAPA), the BioA product 7,8-diaminopelargonic acid
554 (DAPA), and the BioD product dethiobiotin. The total biotin-vitamers in the filtered
555 supernatants were measured basically as described previously (18) using strain Δ*ppc* as
556 an indicator. It is noted that the growth responses of the indicator strain toward KAPA,
557 DAPA, and dethiobiotin was nearly the same as that to biotin at least within a range
558 from 1 to 100 μg per liter. The bioassay plates consisted of two layers per plate: 15 ml
559 of biotin-free MM-bottom agar (1.5%) and 3 ml of biotin-free MM-top agar (0.8%). The
560 MM-top agar was supplemented with 0.1 ml of indicator-cell solution that was prepared
561 as described previously (65). The bioassay plates were loaded with sterilized paper
562 disks supplemented with 100 μl of the filtered supernatants. After overnight culture at
563 30ºC, the halos formed around the disks due to the growth of the indicator strain were
564 measured.
565
566 **Pimelic acid production.** A 3 ml sample of the seed culture grown in BYG medium to
567 the mid-exponential phase at 30ºC was inoculated into a 300-ml baffled Erlenmeyer
568 flask containing 30 ml of LFG1 medium, followed by cultivation at 30ºC using a rotary
569 shaker at 200 rpm. After glucose was consumed, the culture supernatant was prepared
570 by removing cells through centrifugation and subsequent filtration with a Millex-MA
571 filtrate unit. The resulting solution was subject to LC-MS/MS analysis.
572
573 **Quantitative determination of pimelic acid.** Pimelic acid in culture supernatant was
574 determined using LC-MS/MS system of a Quattro micro API (MS) system with a
575 Waters ACQUITY UPLC (LC) (Waters Co.). Separation was performed at 40ºC using a
576 Chemcobond 5-ODS-W reversed phase column (4.6×250 mm; ChemcoPlus Scientific

577 CO., Ltd., Japan) with isocratic elution. Elution was performed at a flow rate of 0.5 mL
578 min^{-1} using a 0.1% formic acid containing 25% acetonitrile and injection volume was
579 50 µL. The mass spectrometer was operated in negative mode electrospray ionization
580 (ESI^-) with multiple reaction monitoring (MRM). The mass transition ion was selected
581 as *m*/*z* 159.2 → 97.0 for pimelic acid. The other optimized MS/MS parameters were as
582 follows: 3,000 V of capillary voltage, 25V of cone voltage, 600 liter h^{-1} of desolvation
583 gas (N_2) flow, 50 liter h^{-1} of cone gas (N_2) flow, 120ºC of source temperature, 350ºC of
584 desolvation temperature, 9.0 ml h^{-1} of argon gas flow (Ar), and 15 V collision voltage.
585 The analytical conditions were determined in preliminary experiments. A linear standard
586 curve was obtained using pimelic acid at a concentration range from 10 to 1,000 µg
587 $liter^{-1}$.
588
589 **Liquid cultures to examine the phenotypes of *fas* disruption.** A 1 ml sample of the
590 seed culture grown in BYG medium to the mid-exponential phase was harvested,
591 resuspended in 1 ml of saline, and then diluted 10 times with saline. The main culture
592 was started by inoculating 0.005 ml of the 10-times diluted seed culture into 5 ml of
593 MM medium supplemented with biotin at a final concentration of 100 µg per liter. In
594 this experiment, the final inoculum size from the seed culture to the main culture
595 corresponds to 0.01%. When required, sodium oleate, lipoic acid, or octanoic acid were
596 added at the indicated concentrations. All liquid cultures were incubated at 30ºC in
597 L-type test tubes on a Monod shaker at 48 strokes per min. To minimize the influence of
598 the carryover of lipoic acid or other unnecessary nutrients, β-cyclodextrin (Nacalai
599 Tesque, Kyoto, Japan) with clathrate action toward lipoic acid (68) was added to the
600 MM medium to 1.5%.

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FIGURE LEGENDS

FIG 1 Proposed *de novo* biosynthetic pathways and the relevant genes of biotin and lipoic acid in *C. glutamicum*. Fatty acid biosynthesis in this organism begins with the reaction of acetyl-CoA carboxylase consisting of three subunits AccBC, AccD1, and AccE, and then proceeds to the FAS-I pathway consisting of FasA and FasB. The biotin-biosynthetic pathway of *C. glutamicum* is incomplete due to the lack of *bioF* and the gene for the *de novo* synthesis of pimeloyl-CoA (or -ACP). In the previous study, we demonstrated that *E. coli bioBF* and *B. subtilis bioI* could bridge the gaps (18). The origin of pimeloyl-CoA (or -ACP) *in vivo* could be the fatty acid-biosynthetic pathway, but this remains speculative. In contrast, lipoic acid is assumed to be synthesized from octanoyl-CoA (or -ACP) in a manner similar to that of *E. coli* (3). The octanoyl moiety is first transferred to the apoprotein (E2) by LipB, and is then converted to lipoic acid by LipA to form lipoyl-E2. Also in this case, the origin of octanoyl-CoA (or -ACP) remains an enigma.

FIG 2 Schematic diagram of the creation of strain *C. glutamicum* BFI-5 carrying *E. coli*

817 *bioBF* and *B. subtilis bioI* on its genome. We previously constructed *C. glutamicum*
818 BF-3, in which the *E. coli* genomic region comprising the *bioBF* gene cluster and its
819 promoter/operator sequence (*P/O*) was inserted into the wild-type genome (18).
820 Likewise, the *B. subtilis bioI* gene was inserted in the vicinity of the *bioBF* genes so as
821 to be constitutively expressed under the promoter (P_{gapA}) of the endogenous *gapA* gene.
822
823 **FIG 3** Biotin-vitamer production by strain BFI-5 with disrupted fatty acid biosynthesis
824 genes (A) and amplified fatty acid biosynthesis genes (B). Cultivations were carried out
825 in 30 ml of biotin-free MM medium (1% glucose) supplemented with10 μg of lipoic
826 acid per liter in 300-ml baffled Erlenmeyer flasks. For cultures of strains BFIΔ*fasA* and
827 BFIΔ*fasAB*, 1 g of Tween 80 per liter was added to satisfy the oleic auxotrophy. The
828 control strain BFI-5 and strain BFIΔ*fasB* were cultivated under the conditions both with
829 (+) and without (-) Tween 80 (1 g $liter^{-1}$). Plasmid carriers were cultivated in the
830 presence of 10 mg of kanamycin per liter. Under these conditions, the plasmid
831 maintenance rate at the end of cultivation was more than 97.0% in all cultures. Titers of
832 biotin-vitamers are means and standard deviations of three independent cultures.
833 Growth values (■) are means of three independent cultures, which showed <5%
834 differences among them. Data for comparison between groups of the control vector
835 carriers and the pCfasB carriers (*) were analyzed by Student's *t* test using JMP
836 statistical software version 8.0.1 (SAS Institute, Cary, NC), and the differences were
837 considered statistically significant at *p* values of <0.03.
838
839 **FIG 4** Growth responses of wild-type strain ATCC 13032 and its *fasA*- and
840 *fasB*-disrupted strains, WTΔ*fasA* and WTΔ*fasB*, respectively, to oleate, lipoic acid, and

octanoic acid. (A) After appropriate dilutions of the cultures, an aliquot (approximately 10^3 cells) was spread onto biotin (100 μg liter^{-1})-supplemented MM agar plates with and without 100 mg of sodium oleate, 10 μg of lipoic acid, or 1 mg of octanoic acid per liter, and cultured at 30ºC for 2 days. The pictures show one representative result of three independent experiments. (B) Cultivations were carried out at 30°C in biotin (100 μg liter^{-1})-supplemented MM liquid culture with none (○), 50 mg of sodium oleate (▲), 50 mg of sodium oleate plus 10 μg of lipoic acid (Δ), 100 mg of sodium oleate (♦), 100 mg of sodium oleate plus 10 μg of lipoic acid (◊), 10 μg of lipoic acid (■), or 1 mg of octanoic acid (□) per liter. The inoculum size from the seed culture to the main culture corresponds to 0.01%, as indicated in Materials and Methods. Values are means of three independent cultures, which showed <5% differences among them.

FIG 5 Growth of strains WTΔ*fasB* carrying the vector plasmid pCS299P, pCfasB, and pCfasA. Cultivations were carried out at 30°C in biotin (100 μg liter^{-1})-supplemented MM liquid culture with none (○), 10μg of lipoic acid (■), or 1 mg of octanoic acid (□) per liter. The inoculum size from the seed culture to the main culture corresponds to 0.01%. The plasmid maintenance rate at the end of cultivation was more than 97.0% in all cultures. Values are means of three independent cultures, which showed <5% differences among them.

TABLE 1 FAS systems in organisms

Organism	Cytosol	Mitochondria	Plastid	Possible sources of the lipoic acid precursor	References
Animals (Mammalians)	FAS-I	FAS-II	—	Mitochondrial FAS-II	46
Plants (Higher plants)	—	FAS-II	FAS-II	Mitochondrial FAS-II Plastidial FAS-II	43, 47, 48
Fungi (*Neurospora crassa*)	FAS-I	FAS-II	—	Mitochondrial FAS-II	49, 50
Yeast (*Saccharomyces cerevisiae*)	FAS-I	FAS-II	—	Mitochondrial FAS-II	41
Escherichia coli	FAS-II	—	—	FAS-II	29
Mycobacterium tuberculosis	FAS-I FAS-II[a]	—	—	unknown	33, 35, 51
Corynebacterium glutamicum	FAS-I	—	—	unknown	31, 33

[a] The mycobacterial FAS-II is thought to be incapable of *de novo* fatty acid synthesis from acetyl-CoA, but it functions in elongating the FAS-I products long-chain fatty acids (12- to 16-carbon) to the very-long chain mycolic acids (35). —, not found.

TABLE 2 Pimelic acid production by strain WTI-1 with amplified fatty acid biosynthesis genes[a]

Strain (Plasmid)	Growth (OD_{660})	Pimelic acid (μg $liter^{-1}$)
ATCC 13032	47.5 ± 1.5	—[b]
WTI-1 (Vector)	47.1 ± 1.7	20.3 ± 0.7
WTI-1 (pCaccBCDE)	46.3 ± 2.1	46.7 ± 2.3
WTI-1 (pCfasA)	46.5 ± 2.0	61.3 ± 2.9
WTI-1 (pCfasB)	46.1 ± 2.2	35.9 ± 1.2

[a] Production was carried out in fermentation medium LFG1 containing 5% glucose in 300-ml baffled Erlenmeyer flasks. After glucose was consumed, the culture supernatants were subject to LC-MS/MS analysis to determine the amounts of pimelic acid. The detection limit of pimeric acid is approximately 1.0 μg $liter^{-1}$ under our analytical conditions. Values are means and standard deviations of three independent experiments.

[b] —, not detected.

TABLE 3 Sequences of primers used in this study

Primer	Sequence[a]	Purpose
accBCPgapAFusF	5'-CCTACAATCTTTAGAGGAGACACAACGTGTCAGTCGAGACTAGGAAGATCACCAAG-3'	Expression of *accBC*
accBCdown100RKpnI	5'-CTTGGTACCGAAATCTTGTTGTCGAATG-3'	Expression of *accBC*
PgapAKpBgF	5'-GCGGGTACCAGATCTGAAGATTCCTGATACAAATTCTGTTG-3'	Expression of *accBC* and *accD1*
accBCPgapAFusR	5'-CTTGGTGATCTTCCTAGTCTCGACTGACACGTTGTGTCTCCTCTAAAGATTGTAGG-3'	Expression of *accBC*
accD1PgapAFusF	5'-CCTACAATCTTTAGAGGAGACACAACATGACCATTTCCTCACCTTTGATTGACGTC-3'	Expression of *accD1*
accD1down150RKpnI	5'-TCGGGTACCGGTTATATTAGCCCAGCG-3'	Expression of *accD1*
accD1PgapAFusR	5'-GACGTCAATCAAAGGTGAGGAAATGGTCATGTTGTGTCTCCTCTAAAGATTGTAGG-3'	Expression of *accD1*
InFu-accBCf	5'-TGAGTCATCAATTTAAATCAGGAGTTATTAATGTCAGTCGAGACTAGGAAG-3'	Expression of *accBC*, *accD1*, and *accE*
InFu-accBCr	5'-TTACTTGATCTCGAGGAGAACAACGC-3'	Expression of *accBC*, *accD1*, and *accE*
InFu-accEf	5'-CTCGAGATCAAGTAAAAACTGTTTTTTAAAGGAGAACCATGTCTGAAG-3'	Expression of *accBC*, *accD1*, and *accE*
InFu-accEr	5'-TATGGATTCGCCGATCTAGAAGAAATTCACATTCTGAAACGCGC-3'	Expression of *accBC*, *accD1*, and *accE*
InFu-accD1f	5'-ATCGGCGAATCCATAAAGGTTCAAAAG-3'	Expression of *accBC*, *accD1*, and *accE*

InFu-accD1r	5’-TAAATTGATGACTCATTACAGTGGCATGTTGCCGTGCTTG-3’	Expression of *accBC*, *accD1*, and *accE*
fasAexpFupEcoRV	5’-TGGGATATCCTGTGGTGGCTTTTAAGAAG-3’	Expression of *fasA*
fasAexpRdownNheI	5’-TGCGCTAGCAAACTTGAGAAGTTTCATGAG-3’	Expression of *fasA*
InVer-pCS299PfasAf	5’-AAGTTTGCTAGCGCAGGCATGCAAGCTTGGCGTAATCATGG-3’	Expression of *fasA*
InVer-pCS299PfasAr	5’-CCACAGGATATCCCATGCAGGTCGACTCTAGAGGATCC-3’	Expression of *fasA*
fasBexpFupBlnI	5’-AGTCCTAGGCCGGGAGCTGTAGAAAATTGC-3’	Expression of *fasB*
fasBexpRDownNheI	5’-GTTGCTAGCACTAAGTTACCCTCGGTGTGAAG-3’	Expression of *fasB*
InVer-pCS299PfasBf	5’-CTTAGTGCTAGCAACGGCATGCAAGCTTGGCGTAATCATG-3’	Expression of *fasB*
InVer-pCS299PfasBr	5’-TCCCGGCCTAGGACTTGCAGGTCGACTCTAGAGGATCC-3’	Expression of *fasB*
CglfasIA5’BglIIF	5’-ACG*AGATCT*ACGCATTCGTAAGTGG-3’	Deletion of *fasA*
CglfasIAFusR	5’-CAACGGATGCACGTGCCAGGAGGACGGTACCGGTTGCACGTGCCTTGGAAAC-3’	Deletion of *fasA*
CglfasIAFusF	5’-GTTTCCAAGGCACGTGCAACCGGTACCGTCCTCCTGGCACGTGCATCCGTTG-3’	Deletion of *fasA*
CglfasIA3’BglIIR	5’-CAG*AGATCT*TAGCTATCTAACGTTTAGC-3’	Deletion of *fasA*
fasBupF	5’-CAGTATTCCTGTGCATGTGAATACGC-3’	Deletion of *fasB*

fasBFusR	5’-AGGAGGACTGCAGCTTCA ACTTCGTTCCTGCTCAATTCG GTCACGT-3’	Deletion of *fasB*
fasBFusF	5’-ACGTGACCGAATTGAGCA GGAACGAAGTTGAAGCTGC AGTCCTCCT-3’	Deletion of *fasB*
fasBdnR	5’-TCTTGATCAAGGTGCCGG TGGGAA-3’	Deletion of *fasB*
ncrFBam2	5’-ACT**GGATCC**ACACATAAG TGCTCT-3’	Integration of *bioI* under the *gapA* promoter into the *C. glutamicum* genome
PgapAFusR	5’-CTAAATTTCTTCCAACAAA TCTTCCGTCTTGTTTCAGGCC ACCACTTAGAAGGC-3’	Integration of *bioI* under the *gapA* promoter into the *C. glutamicum* genome
PgapAFusF	5’-GCCTTCTAAGTGGTGGCCT GAAACAAGACGGAAGATTT GTTGGAAGAAATTTAG-3’	Integration of *bioI* under the *gapA* promoter into the *C. glutamicum* genome
bioIFusR	5’-GCAGTTGACGATGCAATT GTCACGTTGTGTCTCCTCTAA AGATTGTAGG -3’	Integration of *bioI* under the *gapA* promoter into the *C. glutamicum* genome
bioIFusF	5’-CCTACAATCTTTAGAGGAG ACACAACGTGACAATTGCAT CGTCAACTGC-3’	Integration of *bioI* under the *gapA* promoter into the *C. glutamicum* genome
bioIFusR2	5’-GACAATTGAATTACGCCCT AGTAGTAGATGTTCACTCCC CTTTTTTATAG-3’	Integration of *bioI* under the *gapA* promoter into the *C. glutamicum* genome
ncrFusF	5’-CTATAAAAAAGGGGAGTG AACATCTACTACTAGGGCGT AATTCAATTGTC-3’	Integration of *bioI* under the *gapA* promoter into the *C. glutamicum* genome
ncrRBam2	5’-TAC**GGATCC**CAGCATCAT GCTTGT-3’	Integration of *bioI* under the *gapA* promoter into the *C. glutamicum* genome

[a]KpnI sites are underlined, BglII sites are italicized, BclI site is double-underlined, and BamHI sites are in bold.

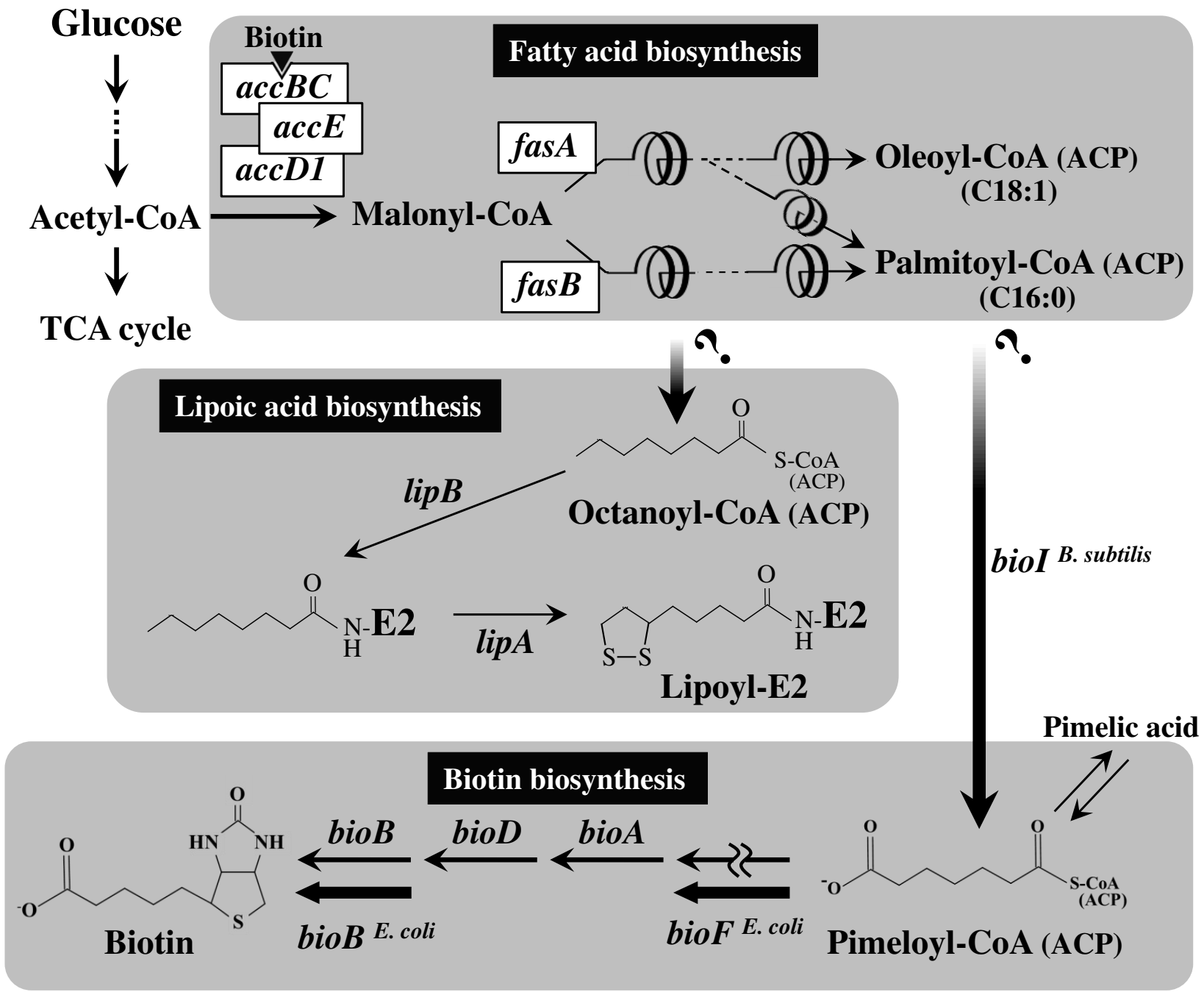

FIG 1 Ikeda et al.

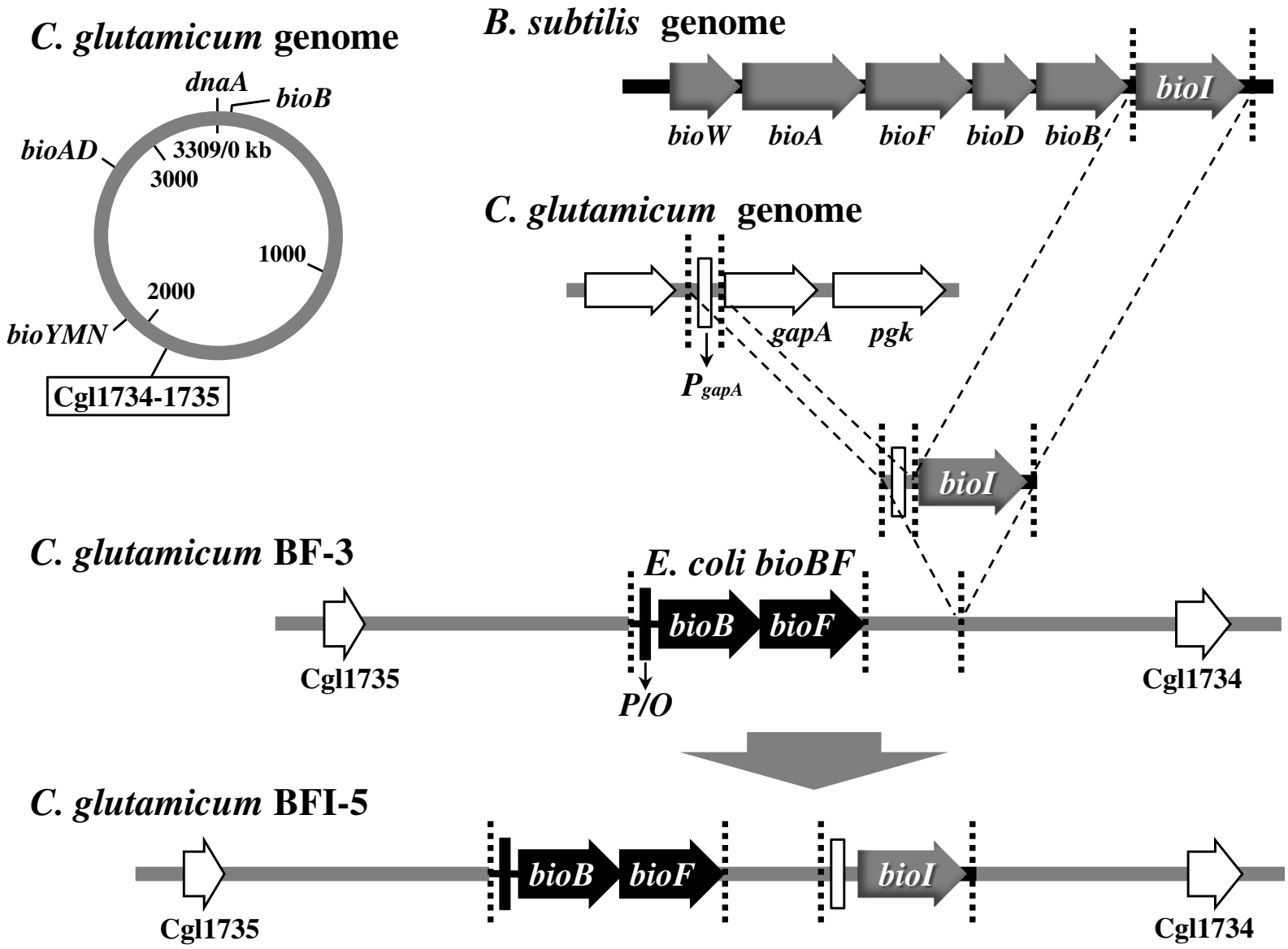

FIG 2 Ikeda et al.

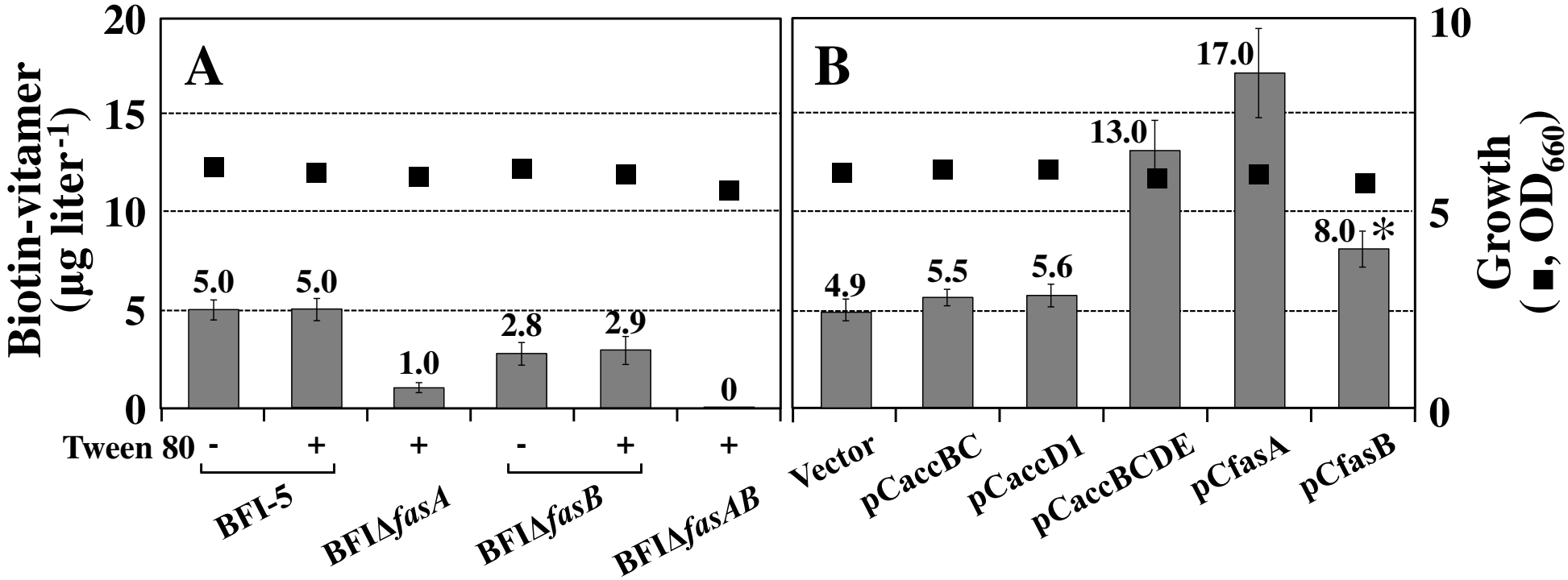

FIG 3 Ikeda et al.

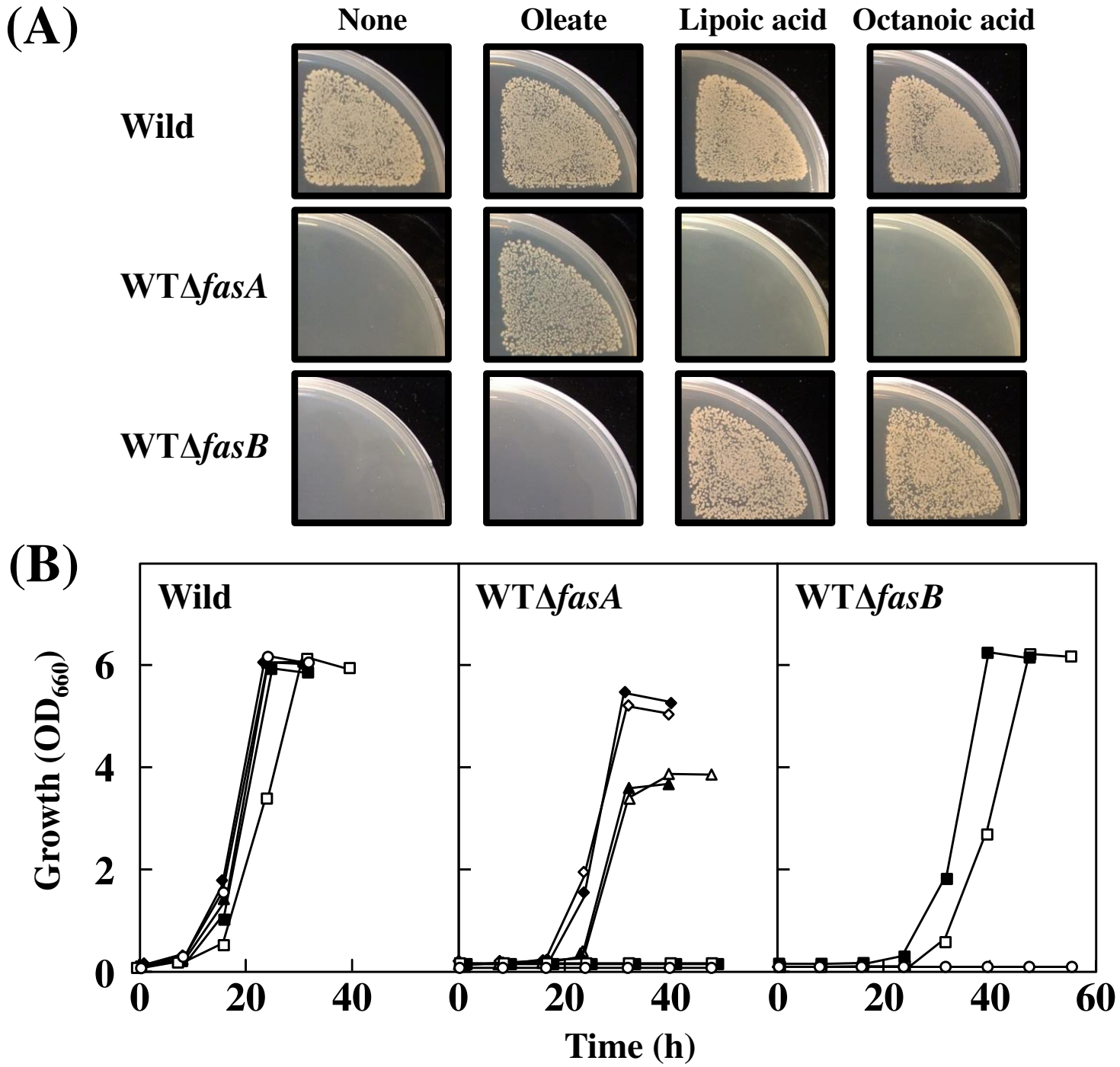

FIG 4 Ikeda et al.

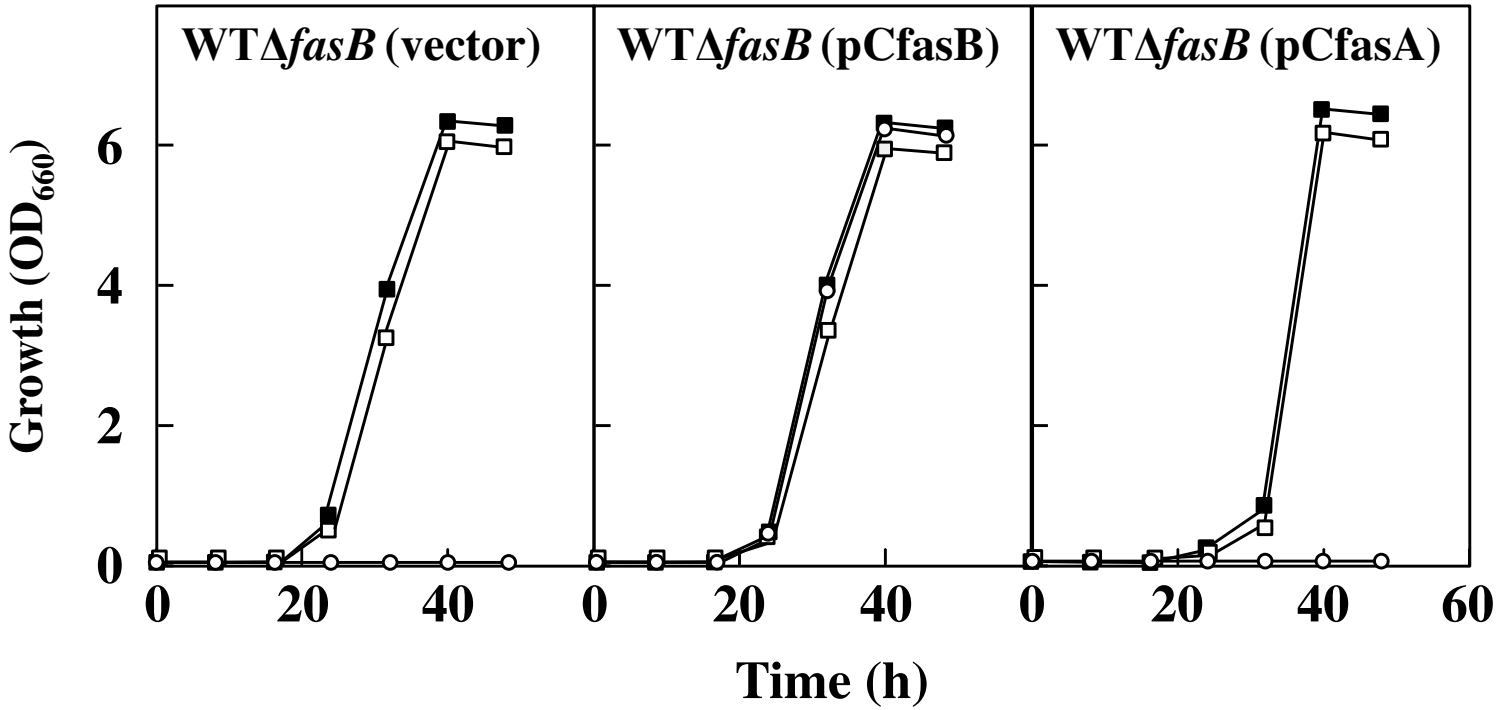

FIG 5 Ikeda et al.