A Futile Metabolic Cycle of Fatty Acy1 Coenzyme A (Acr1-CoA) Hydrolysis and Resynthesis in Corynebacterium glutamicum and Its Disruption Leading to Fatty Acid Production

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ABSTRACT Fatty acyl coenzyme A (acyl-CoA) thioesterase (Tes) and acyl-CoA synthetase (FadD) catalyze opposing reactions between acyl-CoAs and free fatty acids. Within the genome of Corynebacterium glutamicum, several candidate genes for each enzyme are present, although their functions remain unknown. Modified expression of the candidate genes in the fatty acid producer WTΔfasR led to identification of one tes gene (tesA) and two fadD genes (fadD5 and fadD15), which functioned positively and negatively in fatty acid production, respectively. Genetic analysis showed that fadD5 and fadD15 are responsible for utilization of exogenous fatty acids and that tesA plays a role in supplying fatty acids for synthesis of the outer layer components mycolic acids. Enzyme assays and expression analysis revealed that tesA, fadD5, and fadD15 were coexpressed to create a cyclic route between acyl-CoAs and fatty acids. When fadD5 or fadD15 was disrupted in wild-type C. glutamicum, both disruptants excreted fatty acids during growth. Double disruption of these genes resulted in a synergistic increase in production. Additional disruption of tesA revealed a canceling effect on production. These results indicate that the FadDs normally shunt the surplus of TesA-generated fatty acids back to acyl-CoAs for lipid biosynthesis and that interception of this shunt provokes cells to overproduce fatty acids. When this strategy was applied to a high-fatty-acid producer, the resulting fadD-disrupted and tesA-amplified strain exhibited a 72% yield increase relative to its parent and produced fatty acids, which consisted mainly of oleic acid, palmitic acid, and stearic acid, on the gram scale per liter from 1% glucose.

IMPORTANCE The industrial amino acid producer Corynebacterium glutamicum has evolved into a potential workhorse for fatty acid production. In this organism, we obtained evidence showing the presence of a unique mechanism of lipid homeostasis, namely, formation of a futile cycle of acyl-CoA hydrolysis and resynthesis mediated by acyl-CoA thioesterase (Tes) and acyl-CoA synthetase (FadD), respectively. The biological role of the coupling of Tes and FadD would be to supply free fatty acids for synthesis of the outer layer components mycolic acids and to recycle their excess to acyl-CoAs for membrane lipid synthesis. We further demonstrated that engineering of the cycle in a high-fatty-acid producer led to dramatically improved production, which provides a useful engineering strategy for fatty acid production in this industrially important microorganism.

KEYWORDS Corynebacterium glutamicum, mycolic acid-containing bacterium, acyl-CoA thioesterase, acyl-CoA synthetase, futile cycle, fatty acid production

Microbial production of fatty acids, lipids, and their related compounds has received significant attention as a renewable source of biofuels and functional nutrients (1). The fermentative processes have been developed using oleaginous fungi,
yeasts, and algae (2–4). However, attempts to use naturally nonoleaginous bacteria for that purpose have been increasing in recent years (5–7). For example, it has been demonstrated with *Escherichia coli* that cytosolic expression of the periplasmic enzyme acyl-acyl carrier protein (acyl-ACP) thioesterase I (TesA) in cells deficient in the β-oxidative fatty acid degradation pathway resulted in the extracellular production of free fatty acids (5), which has recently become a common strategy for fatty acid production by *E. coli* (8).

Our group and others have developed the amino acid-producing microorganism *Corynebacterium glutamicum* as another platform for the production of fatty acids and their related compounds (9–14). With respect to fatty acid metabolism, this organism has at least three inherent properties that are distinct from those of *E. coli* (Fig. 1): (i) the presence of a eukaryotic multifunctional type I fatty acid synthase (FAS-I) system comprising FasA and FasB (15, 16), in contrast to *E. coli*, which employs an individual nonaggregating type II fatty acid synthase (FAS-II) system (17); (ii) a lack of the β-oxidation pathway involving fatty acid degradation (10, 18); and (iii) a high level of cytoplasmic acyl coenzyme A (acyl-CoA) thioesterase (Tes) activity (10). Owing to these unique *C. glutamicum* properties, only a loss-of-function mutation of *fasR*, which encodes a fatty acid biosynthesis repressor protein (19), gives rise to fatty acid production without modification to the Tes enzyme (10). This, however, raises the question of why this organism has naturally high cytoplasmic Tes activity, in contrast to *E. coli*.

*C. glutamicum* is likely to possess a unique mechanism that differs from that of *E. coli* for maintaining lipid homeostasis. This prompted us to focus on Tes and, at the same time, its opposing enzyme acyl-CoA synthetase (FadD), which catalyzes the activation of free fatty acids to acyl-CoAs using ATP (Fig. 1).

With respect to the corresponding two enzymes in *E. coli*, considerable information is available regarding the enzymatic and functional properties as well as the genes. For Tes, three isoforms, Tes I, II, and III, have so far been identified (20, 21). Tes I, encoded by *fasR*, plays a critical role in fatty acid biosynthesis and is repressed by the fatty acid synthase (FAS-I) complex at the transcriptional level (20). Tes II and Tes III are involved in the synthesis of specific fatty acids, such as palmitic acid (21). FadD, encoded by *fasO*, is responsible for the activation of free fatty acids to acyl-CoAs and is regulated by both the metabolic status of the cell and the concentration of acyl-CoAs (22). The regulation of Tes and FadD is crucial for the efficient production of fatty acids and their related compounds.

**Fig 1** Lipid metabolism and its predicted regulatory mechanism in *C. glutamicum*. For fatty acid biosynthesis, *C. 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 products of the FAS-I pathway are believed to be acyl-CoAs (51), which are used for the synthesis of membrane phospholipids and the outer layer components mycolic acids. FasB is also involved in the synthesis of octanoic acid (C₈), a precursor of lipoic acid (12). This organism is naturally unable to degrade fatty acids, since three genes (gray arrows) responsible for the β-oxidation of fatty acids are missing from the *C. glutamicum* genome (18). Based on knowledge of related bacteria, acyl-CoAs are thought to inhibit Acc, FasA, and FasB (52, 53). The repressor protein FasR, combined with the effector acyl-CoAs, represses the expressions for accD₁, fasA, and fasB (19, 54). Repression and predicted inhibition are indicated by double lines. Arrows with solid and dotted lines represent single and multiple enzymatic processes, respectively. FadE, acyl-CoA dehydrogenase; EchA, enoyl-CoA hydratase; FadB, hydroxyacyl-CoA dehydrogenase; FadD, ketoacyl-CoA reductase; CM, cytoplasmic membrane; OL, outer layer.
by tesA, is specific for C_{12} to C_{18} acyl-thioesters, while it is unlikely to have access to acyl-thioesters, because native Tes I is a periplasmic enzyme (22). Thus, the physiological role of this enzyme remains unknown. A second Tes II, a cytosolic enzyme encoded by tesB, has broader substrate specificity and is active for C_{8} to C_{18} acyl-thioesters. However, since no obvious physiological or biochemical defect was observed in E. coli with tesB overexpression or deletion (23, 24), the exact physiological function of Tes II in lipid metabolism has not been established. A third Tes III, which is encoded by fadM (ybaW), has been shown to be a long-chain acyl-CoA thioesterase that is most active with 3,5-tetradecadienoyl-CoA, a minor metabolite of the β-oxidation of oleic acid (21). Although the fadM gene is not essential for growth, it has been demonstrated to be inductively expressed when E. coli is grown on oleic acid as the sole carbon source (21, 25). Based on these findings, Tes III has been assumed to perform a backup role in fatty acid β-oxidation.

On the other hand, E. coli FadD is a single enzyme encoded by fadD and has a broad substrate specificity for fatty acids of medium to long chain lengths (26), although E. coli has another enzyme, FadK, that is involved in the activation of short-chain fatty acids solely under anaerobic conditions (27). The fadD gene has been reported to be derepressed after cells enter the stationary phase of growth, where free fatty acids are generated from the degradation of membrane lipids (28). Thus, the physiological role of E. coli FadD is to activate fatty acids that originated from membrane lipids to generate acyl-CoAs, which are further metabolized by the β-oxidation pathway to generate a source of carbon and energy (28).

The C. glutamicum genome indicates the presence of two and five putative genes for Tes and FadD, respectively, although the functions of most remain unclear. In this study, we aimed to (i) identify the tes and fadD genes involved in fatty acid production by C. glutamicum and (ii) clarify the roles of each gene in fatty acid metabolism and production. Here, we obtained evidence showing that the relevant Tes and FadD enzymes form a futile metabolic cycle of acyl-CoA hydrolysis and resynthesis during growth on glucose, providing a new mechanism for maintaining both lipid homeostasis and fatty acid production. Why does this organism need to employ such a unique futile cycle? Its physiological significance is discussed below.

**RESULTS**

*In silico analysis of candidate genes for FadD and Tes.* The C. glutamicum genomic database (GenBank accession number BA000036) indicates the presence of the following five genes encoding putative FadD proteins (see Fig. S1A in the supplemental material): fadD1 (Cgl0284, NCgl0279), fadD5 (Cgl0400, NCgl0388), fadD4 (Cgl1198, NCgl1151), fadD15 (Cgl2296, NCgl2216), and fadD32 (Cgl2872, NCgl2774). All of these genes contain ATP/AMP and fatty acyl-CoA synthetase (FACS) motifs homologous to those in E. coli FadD (Fig. S1B) (29). To date, however, there have been no reports on their functions, except for fadD32, which is located in a chromosomal cluster with accD3 and pks (Fig. S1A) and has been assumed to be involved in the synthesis of mycolic acids by activating free fatty acids to form acyl-AMP (14, 30). On the other hand, C. glutamicum possesses two annotated tes genes, tesB (Cgl1664, NCgl1600) and tesA (Cgl2451, NCgl2365), whose functions remain to be clarified (Fig. S2A). The deduced amino acid sequence of the tesA product, unlike that of the tesB product, has an active-site motif that is homologous to the E. coli ybaW product Tes III, a long-chain acyl-CoA thioesterase (Fig. S2B) (21), suggesting the involvement of tesA in the hydrolysis of long-chain fatty acyl-CoAs.

*In vivo identification of the genes responsible for the conversion between long-chain fatty acids and their CoA derivatives.* Our first task was to identify the fadD and tes genes responsible for the transfer and release, respectively, of CoA between long-chain fatty acids and their CoA derivatives. Based on the catalytic reactions, it was reasonable to expect that the intended fadD gene negatively affects fatty acid production when amplified in a fatty acid producer while the intended tes gene plays a pivotal role in fatty acid production. Initially, to identify the types of fadD genes
present among the five candidate fadD genes, their coding regions were individually cloned on a multicopy vector so as to be constitutively expressed under the control of the promoter of the endogenous gapA gene, encoding glyceraldehyde 3-phosphate dehydrogenase, to generate plasmids pCFadD1, pCFadD4, pCFadD5, pCFadD15, and pCFadD32. Each plasmid was introduced into the C. glutamicum fatty acid-producing strain WTΔfasR, and the resulting plasmid carriers were compared with the control vector carrier for fatty acid production when cultivated in minimal medium (MM) (1% glucose). As shown in Table 1, plasmids pCFadD5 and pCFadD15 brought about approximately 25% and 28% decreases in fatty acid production, respectively, while the other three plasmids had only marginal or relatively small effects on production. These data suggest that fadD5 and fadD15 play significant roles in getting free fatty acids back to their CoA derivatives. Although the pCFadD32 carrier showed a 15% decrease in production, we believed that this was probably due to the redirection of carbon into mycolic acid synthesis, considering the predicted role of FadD32, namely, activation of myo-inositol. The resulting strain, WTΔfasRtesA, grew well regardless of the carbon sources and exhibited a \( \text{OD}_{600} \) of 1.02, indicating that the loss of tesA function should impair the ability of strain WTΔfasR to produce fatty acids.

As for tes, the above-mentioned \textit{in silico} analysis suggested that the gene annotated as tesA is a more likely candidate for long-chain fatty acyl-CoA hydrolysis than the other tesB gene. If so, the loss of tesA function should impair the ability of strain WTΔfasR to produce fatty acids. In fact, disruption of tesA resulted in an almost complete loss of fatty acid production ability, and this phenotype was fully complemented by the plasmid-mediated expression of tesA (Table 1). However, since the tesA-disrupted strain WTΔfasRtesA showed an approximately 40% lower growth level than its parent, WTΔfasR, as can be seen from the optical density (OD) (Table 1), there remained a possibility that the reduced growth might affect fatty acid production. To avoid this, we expressed the chromosomal tesA gene under the control of the myo-inositol-inducible promoter of \textit{iolT1}, encoding a myo-inositol transporter in strain WTΔfasR, followed by examination for fatty acid production under the conditions with glucose and with myo-inositol. The resulting strain, WTΔfasRtesA\textit{iol}, grew well regardless of the carbon sources and exhibited a myo-inositol-dependent fatty acid-producing phenotype, while the control WTΔfasR strain produced fatty acids under both conditions (Table 1). These data strongly suggest that tesA is responsible for long-chain fatty acyl-CoA hydrolysis and is thus essential for fatty acid production.

**Significance of fadD5 and fadD15 in oleic acid utilization.** Naturally biotin-auxotrophic C. glutamicum can grow on glucose even under biotin-free conditions, provided that oleic acid is used to supplement the medium (31). This is because exogenous oleic acid is activated to oleoyl-CoA by FadD and is then utilized for membrane

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**TABLE 1** Effect of modified fadD or tesA expression on fatty acid production by strain WTΔfasR

| Strain (plasmid)       | Carbon source | Growth (OD\textsubscript{600}) | Fatty acid concn (mg/liter)\textsuperscript{a} |
|------------------------|---------------|---------------------------------|---------------------------------------------|
|                        |               |                                 | Oleic acid | Palmitic acid | Stearic acid | Total         |
| WTΔfasR (vector)       | Glucose       | 8.2 ± 0.4                       | 91.4 ± 3.3 | 73.0 ± 2.5   | 7.6 ± 0.6 | 172.0 ± 6.3 (1.0) |
| WTΔfasR (pCFadD1)      | Glucose       | 8.0 ± 0.3                       | 83.7 ± 2.2 | 71.3 ± 4.3   | 3.0 ± 0.8 | 157.9 ± 7.1 (0.92) |
| WTΔfasR (pCFadD4)      | Glucose       | 7.8 ± 0.3                       | 89.4 ± 1.7 | 72.2 ± 0.9   | 9.5 ± 0.4 | 171.2 ± 2.9 (1.0) |
| WTΔfasR (pCFadD5)      | Glucose       | 8.1 ± 0.6                       | 64.5 ± 6.7 | 57.1 ± 4.4   | 7.4 ± 1.0 | 129.1 ± 11.9 (0.75) |
| WTΔfasR (pCFadD15)     | Glucose       | 8.0 ± 0.7                       | 66.3 ± 6.8 | 50.5 ± 4.7   | 7.1 ± 1.6 | 124.1 ± 13.2 (0.72) |
| WTΔfasR (pCFadD32)     | Glucose       | 8.2 ± 0.6                       | 74.2 ± 5.0 | 63.2 ± 4.8   | 7.6 ± 1.9 | 145.5 ± 11.5 (0.85) |
| WTΔfasRtesA            | Glucose       | 5.2 ± 0.3                       | 2.3 ± 0.3  | 1.6 ± 0.2    | 0.2 ± 0.04 | 4.1 ± 0.4 (0.02) |
| WTΔfasRtesA (pCtesA)   | Glucose       | 8.1 ± 0.5                       | 107.2 ± 7.1 | 78.6 ± 3.1  | 9.7 ± 0.7 | 195.5 ± 10.6 (1.14) |
| WTΔfasR                | Glucose       | 8.6 ± 0.4                       | 90.2 ± 4.5 | 78.7 ± 3.1   | 6.7 ± 0.4 | 175.6 ± 8.0 (1.02) |
|                       | myo-inositol  | 8.0 ± 0.5                       | 97.3 ± 5.1 | 64.6 ± 3.4   | 7.7 ± 0.7 | 169.6 ± 8.9 (0.99) |
| WTΔfasRtesA\textit{iol} | Glucose      | 8.5 ± 0.4                       | 3.6 ± 0.2  | 2.1 ± 0.2    | 0.3 ± 0.03 | 6.0 ± 0.4 (0.03) |
|                        | myo-inositol  | 8.3 ± 0.6                       | 94.4 ± 3.9 | 69.7 ± 2.7   | 4.7 ± 0.5 | 168.8 ± 5.3 (0.98) |

\textsuperscript{a}Production was carried out using 300-ml baffled Erlenmeyer flasks containing 30 ml of MM (1% glucose or 1% myo-inositol). After glucose or myo-inositol was consumed, total lipids, including free fatty acids, were extracted from the culture supernatant to determine free fatty acids by gas chromatography. Value means and standard deviations of the results from three independent experiments. Values in parentheses are relative to the titer obtained with the control strain, WTΔfasR (vector).
lipid biosynthesis. Accordingly, based on the above-mentioned results, it is reasonable to expect that the loss of \textit{fadD5} and/or \textit{fadD15} functions would result in impaired growth even in the presence of oleic acid under biotin-free conditions. To verify this, we constructed \textit{fadD5}- and \textit{fadD15}-disrupted strains, designated WT\textit{D}fadD5 and WT\textit{D}fadD15, respectively, and their double disruptant, designated WT\textit{D}fadD5&15, from the wild-type strain. As shown in Fig. 2A, the wild-type strain (WT) grew normally under biotin-free and oleate-supplemented conditions, while strains WT\textit{D}fadD5 and WT\textit{D}fadD15 exhibited the expected phenotypes of retarded growth under the same culture conditions. In the case of strain WT\textit{D}fadD5&15, no growth was observed, although the presence of biotin in the culture restored the growth. We further confirmed that the inability of strain WT\textit{D}fadD5&15 to utilize exogenous oleate was complemented, although only partially, by the plasmid-mediated expression of \textit{fadD5} or \textit{fadD15} (Fig. 2B). These results fortify our earlier conclusion that \textit{fadD5} and \textit{fadD15} play a role in converting long-chain fatty acids to their CoA derivatives.

\textbf{Significance of the \textit{tesA} gene in growth.} Although the data discussed above indicate the essential role of the genomic \textit{tesA} gene in fatty acid production, its physiological function remains to be clarified. Thus, we examined the phenotype of deficiency in \textit{tesA} under the wild-type background. As shown in Fig. 3A, the \textit{tesA}-disrupted strain WT\textit{D}tesA showed impaired growth on glucose, and this phenotype was almost fully recovered under the conditions of supplementation with either oleate or palmitate. To further confirm this, we expressed chromosomal \textit{tesA} under the control of the \textit{myo}-inositol-inducible promoter of \textit{iolT1}. When the resulting strain, WTtesA\textit{iol}, was cultivated on 1% glucose or 0.5% glucose plus 0.5% \textit{myo}-inositol, it exhibited \textit{myo}-inositol-dependent growth (Fig. 3B). These results indicate that the \textit{C. glutamicum} wild type requires the \textit{tesA} function for normal growth on glucose and its deficiency causes the requirement for the free fatty acid oleate or palmitate.

\textbf{Enzymatic activities and transcript levels in glucose-grown cells.} The results so far described suggest that \textit{fadD5}, \textit{fadD15}, and \textit{tesA} are all expressed in wild-type cells grown on glucose. However, if TesA and FadDs operate simultaneously, a futile cycle that leads to the hydrolysis of ATP as the net effect would result (Fig. 1), which would be unreasonable. Therefore, we examined whether the coexpression actually occurred in wild-type cells by measuring the enzymatic activities and transcript levels. Initially, we determined the enzymatic activities using soluble fractions prepared from cells in...
the late exponential phase of growth on glucose. As for FadD, although significant activity was detected in wild-type cells (11.0 ± 0.4 mU/mg of protein), double disruption of fadD5 and fadD15 resulted in activity being reduced to a marginal level (1.6 ± 0.9 mU/mg). The fadD5- and fadD15-disrupted strains showed moderate levels of activity (5.5 ± 0.3 mU/mg and 7.1 ± 0.4 mU/mg, respectively). On the other hand, a relatively high level of Tes activity was detected in wild-type cells (276 ± 53 mU/mg), in contrast to a trace level in the tesA-disrupted strain.

Next, we investigated the transcript levels of fadD5, fadD15, and tesA in the wild-type strain and its derived fadD5-, fadD15-, fadD5- and fadD15-, and tesA-disrupted strains during growth on glucose (Fig. S3). The data for the three genes in each disruptant are presented as relative to values obtained for the corresponding genes in the wild-type strain. The transcript level of each gene was shown to fall to a negligible level as a consequence of the disruption of the corresponding gene, indicating that the three genes are all expressed in wild-type cells grown on glucose.

This series of data not only reconﬁrmed our earlier conclusion that the intended Tes and FadD activities are speciﬁed by tesA and the two fadD genes (fadD5 and fadD15), respectively, but also veriﬁed that the two opposing reactions operate simultaneously in wild-type cells during growth on glucose.

Effects of fadD5 and fadD15 disruption on fatty acid production. The ﬁndings that tesA and the two fadD genes were coexpressed in wild-type cells during growth on glucose suggest the formation of a cyclic metabolic route between long-chain fatty acids and their CoA derivatives. If so, and considering that this organism naturally lacks the β-oxidation pathway involving fatty acid degradation, it is likely that blockage of the cycle at the FadD step would provoke cells to accumulate fatty acids which are normally synthesized by this organism, namely, oleic acid, palmitic acid, and stearic acid. Furthermore, the potential to accumulate the fatty acids should be cancelled by the additional disruption of tesA. Based on these assumptions, we examined whether the disruption of fadD5 and/or fadD15 causes fatty acid production in wild-type cells during cultivation in MM with 1% glucose. As shown in Fig. 4A, signiﬁcant amounts of fatty acids were found to accumulate in the cultures of the fadD5-disrupted and fadD15-disrupted strains WTΔfadD5 and WTΔfadD15, respectively, whereas the control wild type produced no detectable fatty acids. When both fadD5 and fadD15 were disrupted in wild-type cells, the resulting double disruptant, WTΔfadD5&15, exhibited synergistically increased production under the same conditions.

Next, we examined whether tesA played a key role in fatty acid production by strain WTΔfadD5&15. As shown in Fig. 4B, the disruption of tesA in strain WTΔfadD5&15 led to an almost complete loss of fatty acid production ability concomitantly with a loss of Tes activity, while plasmid-mediated ampliﬁcation of tesA in the triple disruptant WTΔfadD5&15ΔtesA resulted in dramatically increased production with increased Tes
activity. It is noteworthy that when tesA was amplified in the wild-type strain, fatty acid production was not observed (data not shown).

To further confirm that fatty acid production by strain WTΔfadD5&15 occurred during growth on glucose and not after cells entered the stationary phase, we examined its fermentation profile using the typical fatty acid producer WTΔfasR as a control. As shown in Fig. 5A, fatty acid production by strain WTΔfadD5&15 occurred mainly in the late exponential phase of growth on glucose and was stopped after glucose was consumed, which was almost the same profile as that for the control strain WTΔfasR (Fig. 5B).

These results reinforce our conclusions that TesA and the two FadDs normally form a cycle between long-chain fatty acids and their CoA derivatives (Fig. 1) and that overproduction of fatty acids results when the cycle is blocked at the FadD step.

**Engineering a high-fatty-acid producer through disrupted fadD genes and amplified tesA.** As mentioned above, engineering of the metabolic cycle consisting of TesA and FadDs, specifically, a combination of disrupted fadD5 and fadD15 and amplified tesA (referred to as the TesFad method), allows *C. glutamicum* to produce a significant amount of fatty acids, even on a wild-type background (Fig. 4B). The TesFad method differs in production mechanism from the general method, namely, deregulation of fatty acid synthesis, and is expected to be a new strategy for fatty acid produc-

**FIG 4** Fatty acid production by fadD disruptants (A) and the double disruptant WTΔfadD5&15 with disrupted and amplified tesA (B). All cultivations were carried out in 30 ml of MM (1% glucose) in 300-ml baffled Erlenmeyer flasks. After glucose was consumed, total lipids, including free fatty acids, were extracted from the culture supernatant to determine levels of free fatty acids by gas chromatography. Specific activities of Tes in strains WTΔfadD5&15 with disrupted and amplified tesA are also shown (B). Values are means and standard deviations of the results from three independent experiments.

**FIG 5** Fermentation profiles by strains WTΔfadD5&15 (A) and WTΔfasR (B). Two different lines of fatty acid producers, WTΔfadD5&15 and WTΔfasR, were cultivated in 300-ml baffled Erlenmeyer flasks containing 30 ml of MM (1% glucose). Levels of free fatty acids, as well as growth and residual glucose, were measured every 6 h. Values are means and standard deviations of the results from three independent experiments.
tion in C. glutamicum. To substantiate the method, we applied it to our best fatty acid producer, PCCA-3 (14). This producer was developed by assembling four positive mutations (fasR20, fasA63up, fasA2623, and accD3A433T) in the wild-type genome, and it has the capability of producing approximately 600 mg/liter of fatty acids, which consist mainly of oleic acid (449 mg/liter), palmitic acid (133 mg/liter), and stearic acid (40 mg/liter), in flask cultivation with 1% glucose (Fig. 6). Using this strain as a host, we examined the effects of the TesFad method on fatty acid production. As shown in Fig. 6, disruption of the two fadD genes alone (designated strain PCCA-3fadD5&15) or plasmid-mediated amplification of tesA alone (designated strain PCCA-3(pCtesA)) had relatively small effects on production, but their combination (resulting in strain PCCA-3fadD5&15(pCtesA)) resulted in significantly increased production that achieved a titer of 1,071 mg/liter with a conversion yield of approximately 10% on glucose. These data demonstrate that the effects of the TesFad method are not offset by the performance that strain PCCA-3 has already acquired, and the method could therefore be a useful addition to strain improvement for fatty acid production in this organism. It should be noted that, despite a large increase in the total amount of fatty acids, the fatty acid composition of oleic acid, palmitic acid, and stearic acid remained substantially unchanged when the TesFad method was applied (Fig. 6). It is also noteworthy that no significant by-production of glutamic acid was observed in the engineered strain, suggesting that the engineering would not affect the cell surface structure enough to elicit glutamic acid production.

**DISCUSSION**

In this study, we identified Cgl2451 (tesA) as the tes gene responsible for the hydrolysis of long-chain fatty acyl-CoAs into free fatty acids, as well as two fadD genes, Cgl0400 (fadD5) and Cgl2296 (fadD15), that mediate its opposing reaction in C. glutamicum. Interestingly, disruption of the two fadD genes allowed wild-type C. glutamicum to produce considerable amounts of fatty acids (a mixture of oleic acid, palmitic acid, and stearic acid) during growth on glucose. Since this effect was mostly cancelled by the simultaneous disruption of tesA, the secreted fatty acids are assumed to be generated from acyl-CoAs in a tesA-dependent manner. These results indicate that TesA and the two FadDs normally form a cyclic metabolic route between acyl-CoAs and long-chain fatty acids in this organism (Fig. 1), thus provoking cells to overproduce fatty acids, particularly in situations when the cycle is intercepted at the FadD step. Fatty acid production based on this new metabolic cycle has not yet been reported in the literature.
In certain other bacteria, such as *E. coli* and the nitrogen-fixing bacterium *Sinorhizobium meliloti*, *fadD* mutants accumulate free fatty acids after entering the stationary phase of growth (32). This phenomenon has been explained as follows. Cells grown on carbon sources, such as sugars, usually synthesize acyl-acyl carrier proteins (acyl-ACPs) to build membrane lipids, irrespective of the functions of Tes and FadD. Once the carbon sources are used up, the membrane lipids of cells are subject to phospholipase-mediated hydrolysis to release free fatty acids, which are then activated to acyl-CoAs by FadD and subsequently metabolized by the β-oxidation pathway to generate a source of carbon and energy. In the *fadD* mutants of *E. coli*, the fatty acids cannot be activated to acyl-CoAs and are thus destined to be excreted extracellularly. In contrast, *C. glutamicum* needs to hydrolyze some portion of acyl-CoAs by TesA to supply free fatty acids for the synthesis of the outer layer of mycolic acids. In this context, a surplus of the TesA-generated free fatty acids is recycled to acyl-CoAs by the two FadDs (FadDS/15) for phospholipid synthesis. Therefore, interception of the cyclic metabolic route at the FadD step results in fatty acid overproduction. Although concomitant activities of acyl-CoA hydrolysis and resynthesis create a futile cycle, this has significance as the recycling system of excess fatty acids, especially in an organism that cannot use free fatty acids as a source of carbon and energy.

![Diagram of lipid metabolism in E. coli and C. glutamicum](image)

**FIG 7** Proposed lipid metabolism in *E. coli* (A) and *C. glutamicum* (B). (A) In the case of *E. coli*, cells grown on carbon sources usually synthesize acyl-acyl carrier proteins (acyl-ACPs) to build membrane lipids, irrespective of the functions of Tes and FadD. After entry into the stationary growth phase, membrane lipids of cells are subject to phospholipase-mediated hydrolysis to release free fatty acids, which are then activated to acyl-CoAs by FadD and subsequently metabolized by the β-oxidation pathway to generate a source of carbon and energy. In the case of *fadD* mutants of *E. coli*, the fatty acids cannot be activated to acyl-CoAs and are thus destined to be excreted extracellularly. (B) In contrast, *C. glutamicum* needs to hydrolyze some portion of acyl-CoAs by TesA to supply free fatty acids for the synthesis of the outer layer of mycolic acids. In this context, a surplus of the TesA-generated free fatty acids is recycled to acyl-CoAs by the two FadDs (FadDS/15) for phospholipid synthesis. Therefore, interception of the cyclic metabolic route at the FadD step results in fatty acid overproduction. Although concomitant activities of acyl-CoA hydrolysis and resynthesis create a futile cycle, this has significance as the recycling system of excess fatty acids, especially in an organism that cannot use free fatty acids as a source of carbon and energy.
glucose to generate free fatty acids, which are usually recycled back to acyl-CoAs by FadD5 and FadD15. This hypothesis is supported by the copresence of Tes and FadD activities in glucose-growing wild-type cells. However, the question of why the organism needs to operate TesA during growth on glucose remains. This appears to be related to a unique feature of its cell envelope. This organism has an outer layer of mycolic acids that are synthesized from free long-chain fatty acids as precursors (30). Therefore, some portion of acyl-CoAs need to be hydrolyzed by TesA to supply free fatty acids for mycolic acid synthesis (Fig. 7B). In this context, a surplus of the TesA-generated free fatty acids would be recycled to acyl-CoAs by the FadDs for biosynthesis of membrane lipids. Taken together, the biological role of the coupling of TesA and FadDs would be to supply free fatty acids for the synthesis of the outer layer of mycolic acids and also to recover their excess (Fig. 7B). The free fatty acids observed in our study are the consequence of interrupted fatty acid recycling.

The TesA-FadD cycle that we proposed for C. glutamicum causes the hydrolysis of ATP as the net effect and thus creates a so-called futile cycle. If this is correct, a question arises as to why this organism employs such an energy-wasting cycle in lipid metabolism. It is likely that the best way for this organism to reconcile satisfying the need for free fatty acids for mycolic acid synthesis with saving energy would be to make the TesA enzyme feedback sensitive to free fatty acids. Nevertheless, to the best of our knowledge, no regulatory mechanism such as this has been reported for any Tes enzyme. Against this backdrop, this organism has had no choice but to develop an alternative method. Considering that C. glutamicum naturally lacks the β-oxidative fatty acid degradation pathway, it is most wasteful for this organism to accumulate a surplus of the TesA-generated free fatty acids. Thus, C. glutamicum might have developed the TesA-FadD cycle as a less energy-wasting and more rational system.

In this study, we demonstrated with our C. glutamicum fatty acid producer that interception of the TesA-FadD futile cycle at the FadD step and the concomitant increase in Tes activity led to dramatically improved production (Fig. 6). The conversion yield on glucose exceeded 10% (wt/wt), which is comparable to the yields reported for the typical fatty acid producers of E. coli (7, 8). This indicates not only the usefulness of this engineering strategy but also the potential of this industrial microorganism as a fatty acid producer. As described in the introduction, previous attempts to produce fatty acids using nonoleaginous bacteria, including E. coli and C. glutamicum, have aimed mainly at the deregulation of fatty acid biosynthesis. In contrast, the engineering strategy presented here focuses on the new mechanism of lipid homeostasis found in C. glutamicum. Specifically, the concept of this engineering strategy is not the deregulation of fatty acid biosynthesis but the blockage of the fatty acid-recycling system that is intrinsic to this organism. Medium-chain fatty acids and dicarboxylic acids have also attracted much attention recently (8) and are potential targets for production by this organism. Although much remains to be clarified in regard to the functions of fadD1, fadD4, and tesB, their products are expected to be involved in the synthesis of those compounds, and our future research will examine this further.

**MATERIALS AND METHODS**

**Bacterial strains.** All strains used in this study are derivatives of the C. glutamicum wild-type strain ATCC 13032. The fatty acid producer WT ΔfasR (10) was derived from ATCC 13032 through the in-frame deletion of fasR, which encodes a fatty acid biosynthesis repressor protein. The high-fatty-acid producer PCCA-3 (14) was developed by the so-called “genome breeding” approach (36), in which four specific mutations (fasA2623, fasA2639, fasA2623, and accD538437) were assembled in the ATCC 13032 genome. The former three mutations contribute to the deregulation of fatty acid biosynthesis and the latter one is assumed to impair the function of the gene product AccD3 to diminish mycolic acid biosynthesis. E. coli DH5α was used as a host for DNA manipulation.

**Plasmids.** Plasmid pCS299P (37), a C. glutamicum-E. coli shuttle vector, was used to clone the PCR products. Plasmid pESB30 (37), which is nonreplicative in C. glutamicum, is a vector for gene replacement in C. glutamicum. Plasmids pCfadD1, pCfadD4, pCfadD5, pCfadD15, and pCfadD32 were constructed so that fadD1 (Cgl0284, NCgl0279), fadD4 (Cgl1198, NCgl1151), fadD5 (Cgl0400, NCgl0388), fadD15 (Cgl2296, NCgl2216), and fadD32 (Cgl2872, NCgl2774) were constitutively expressed under the control of the promoter of the endogenous gapA gene. For the construction of pCfadD15, the open
reading frame (ORF) of fadD15 was amplified using primers fadD15sdFusF and fadD15FusR, with wild-type ATCC 13032 genomic DNA as the template. On the other hand, the genomic region comprising the gapA promoter was amplified using primers PgapApKbpBgF and PgapApFadD15sdFusR so that the ribosome-binding site (RBS) sequence for gapA was altered to the consensus RBS sequence proposed for C. glutamicum (38). Similarly, the genomic region comprising the gapA terminator was amplified using primers fadD15TTgapAFusF and TTgapAPkr. These three fragments were fused by PCR, digested with KpnI, and then ligated to KpnII-digested pCS299P to yield pcfadD15.

Plasmids for the other four fadD genes were constructed by replacing the fadD15 ORF on pCfadD15 with the ORF of the corresponding genes, as follows. Plasmid pCfadD15 was linearized by inverse PCR using primers InVer-PgapAsdR and InVer-TTgapAF so as to completely remove only the fadD15 ORF (for convenience, the resulting linear plasmid is referred to here as fragment A). On the other hand, the ORFs of the target genes were amplified using the primer pairs Infu-fadD1F and Infu-fadD1R for fadD1, InFu-fadD4F and InFu-fadD4R for fadD4, InFu-fadD5F and InFu-fadD5R for fadD5, and InFu-fadD32F and InFu-fadD32R for fadD32. The amplified ORFs were individually cloned into fragment A using an In-Fusion HD cloning kit (Clontech Laboratories, Inc., Mountain View, CA, USA) to yield pCfadD1, pCfadD4, pCfadD5, and pCfadD32. For fadD4 on pCfadD4, the native rare start codon GTG was modified to ATG.

Plasmid pCtesA, which contains the tesA gene (Cgl2451, NCgl2365), was constructed as follows. The genomic region comprising tesA and its native promoter (from −1 to −123 bp upstream of tesA) was amplified using primers tesAup120FbamHI and tesAdown70FbamHI. The resulting fragment was digested with BamHI and then ligated to BamHI-digested pCS299P to yield pCtesA.

The sequences of the primers used in this study are listed in Table 2. All primers were designed based on the genomic sequence of C. glutamicum (BA000036) (39), which is publicly available at http://www.genome.jp/kegg genes.html.

Media. Complete BY medium and minimal medium (MM) were used as basal media for the growth of C. glutamicum strains (40). Solid plates were made by the addition of Bacto agar (Difco) to 1.5%. For preparation of MM containing sodium oleate or sodium palmitate, the fatty acid sodium salt was separately autoclaved and then mixed with a magnesium sulfate solution and a solution containing other components to prevent insolubilization of the fatty acid. For cultivation of plasmid carriers, kanamycin was added at a final concentration of 10 mg/liter. For growth of E. coli, Luria-Bertani broth or agar was used.

Recombinant DNA techniques. Standard protocols (41) were used 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 (40). 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 Pagenet SQC-SAL7 377 (Toyobo, Osaka, Japan).

Strain construction. For the chromosomal deletions of fadD5, fadD15, and tesA, plasmids pCfadD5, pCfadD15, and pCtesA, which contained the corresponding genes with internal deletions, were used to replace the wild-type chromosomal genes with the deleted genes. For the construction of pCfadD5, the 5’ and 3’ regions of fadD5 were amplified using the primer pair In-FudD5up760Fw and fadD5delFusRev, and the primer pair fadD5delFusFw and In-FudD5down740Rev, respectively. These two fragments were fused by PCR and then cloned into linearized pESB30, which was obtained by inverse PCR using primers P9L and P10L, to yield plasmid pCfadD5. For fadD15, the 5’ and 3’ regions of fadD15 were amplified using the primer pair In-FudD15up750Fw and fadD15delFusRev and the primer pair fadD15delFusFw and In-FudD15down700Rev, respectively. Two fragments were fused by PCR and then cloned into linearized pESB30, which was obtained by inverse PCR using primers P9L and P10L with an In-Fusion HD cloning kit. The resulting plasmid, pCfadD15, carried the in-frame deleted fadD5 gene, which was shortened from 1,707 to 120 bp. Similarly, for the construction of pCfadD15, the 5’ and 3’ regions of fadD15 were amplified using the primer pair In-FudD15up750Fw and fadD15delFusRev and the primer pair fadD15delFusFw and In-FudD15down700Rev, respectively. These two fragments were fused by PCR and then cloned into linearized pESB30, which was obtained by inverse PCR using primers P9L and P10L, with an In-Fusion HD cloning kit. The resulting plasmid, pCfadD15, carried the in-frame deleted fadD15 gene, which was shortened from 1,848 to 180 bp. For the construction of pCtesA, the 5’ and 3’ regions of tesA were amplified using the primer pair tesAtesAup300FbamHI and tesAdelFusFw and the primer pair tesAdelFusR and tesAdown770FbamHI, respectively. These two fragments were fused by PCR, digested with BamHI, and then ligated to BamHI-digested pESB30 to yield pCtesA. This plasmid carried the in-frame deleted tesA gene, which was shortened from 468 to 117 bp. The defined chromosomal deletion of the individual gene was accomplished using each plasmid via two recombination events as described previously (42).

For the construction of the myo-inositol-dependent tesA-expressing strains WTtesAI1 and WTfasRtesAI1, the tesA-disrupted strains WTtesA and WTfasRtesA, were used as host strains to replace the chromosomal iot1 gene (Cgl0181, NCgl0178), which is expressed under the control of its native myo-inositol-inducible promoter (43), with the tesA gene. For this gene replacement, plasmid pCpioI1-tesA was constructed as follows. The upstream and downstream regions of the iot1 gene ORF (for convenience, these regions are referred to here as fragments B and C, respectively) were amplified by pairs of primers (InFu-iolT1up450F oder TesAfusR and tesA-iolT1down450F oder TesFusRev). The tesA gene was amplified using primers tesAFusForf and tesAFusRevF. Fragment B, the tesA gene, and fragment C were fused stepwise using PCR. The resulting 1.7-kb fragment was cloned using an In-Fusion HD cloning kit into linearized pESB30, which was obtained by inverse PCR using primers P9L and P10L, to yield plasmid pCpioI1-tesA. The defined chromosomal
replacement of *iolT1* with *tesA* in strains WT*tesA* and WT*fasRtesA* resulted in strains WT*tesA* and WT*fasRtesA*, respectively. Although strains WT*tesA* and WT*fasRtesA* lack the *iolT1* gene, both strains can use myo-inositol as a carbon source due to the existence of an additional transporter encoded by *iolT2* (CgI3058, Ncgl2953).
**Fatty acid production.** A 3-ml sample of the seed culture grown in BY medium to the mid-exponential phase at 30°C was inoculated into a 300-ml baffled Erlenmeyer flask containing 30 ml of MM (1% glucose or 1% myo-inositol), followed by cultivation at 30°C using a rotary shaker at 200 rpm. After glucose or myo-inositol was consumed, total lipids, including free fatty acids, were extracted from the culture supernatant as described previously (14). In the case of the high-fatty-acid producers PCCA-3 and its derivatives shown in Fig. 6, total lipids were extracted from the culture broth containing cells, because some of the fatty acids produced were likely to be insolubilized in the broth. The extracted lipids were subject to quantitative determination of free fatty acids by gas chromatography as described previously (14).

**Liquid cultures to examine the abilities to utilize oleic acid.** A 0.05-ml sample of the first-second culture grown in BY medium to the mid-exponential phase was inoculated into 5 ml of biotin-free MM and cultivated for 20 h to deplete biotin in the culture. The resulting second-second culture was harvested, washed three times with saline, and resuspended in 5 ml of biotin-free MM. The main culture was started by inoculating 0.05 ml of the biotin-depleted second-second culture into 5 ml of biotin-free MM supplemented with the indicated concentrations of biotin or sodium oleate (Fig. 2). All liquid cultures were performed at 30°C in L-type test tubes on a Monod shaker at 48 strokes/min.

**Growth test of strains WTtesA and WTtesAiol.** For strain WTtesA, a 0.05-ml sample of the seed culture grown in BY medium supplemented with 1% glucose was inoculated into 5 ml of MM (1% glucose) supplemented with the indicated concentrations of sodium oleate or sodium palmitate (Fig. 3A). For strain WTtesAiol, a 0.05-ml sample of the seed culture grown in BY medium supplemented with 1% glucose or 0.5% glucose plus 0.5% myo-inositol was inoculated into 5 ml of MM containing 1% glucose or 0.5% glucose plus 0.5% myo-inositol. All liquid cultures were performed at 30°C in L-type test tubes on a Monod shaker at 48 strokes/min.

**Enzyme assays.** Cells grown at 30°C to the late exponential phase in a 300-ml baffled Erlenmeyer flask containing 30 ml of MM (1% glucose) were collected by centrifugation at 10,000 × g for 10 min and washed twice with 50 mM Tris-HCl buffer (pH 8.0) and 150 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-NaOH (pH 7.2) for Tes and FadD assays, respectively. The cells were suspended in the corresponding buffer and sonicated on ice for 10 min using a UD-200 ultrasonic disruptor (Tomy Seiko Co., Ltd., Tokyo, Japan). For the Tes assay, cell debris was removed by centrifugation at 10,000 × g for 10 min, and the supernatant was further ultracentrifuged at 100,000 × g for 90 min using an Optima TL ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). Using the resulting supernatant, the Tes activity was spectrophotometrically measured at 30°C by the methods described by Barnes (44) with the assay mixtures shown in Fig. 6, total lipids were extracted from the culture broth containing cells, because some of the fatty acids produced were likely to be insolubilized in the broth. The extracted lipids were subject to quantitative determination of free fatty acids by gas chromatography as described previously (14).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.6 MB.

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REFERENCES

1. Cho IJ, Choi KR, Lee SY. 2020. Microbial production of fatty acids and derivative chemicals. Curr Opin Biotechnol 65:129–141. https://doi.org/10.1016/j.copbio.2020.02.006.

2. Beopoulou A, Nicolaou JM, Gaullard C. 2011. An overview of lipid metabolism in yeasts and its impact on biological processes. Appl Microbiol Biotechnol 90:1193–1206. https://doi.org/10.1007/s00253-011-3212-8.

3. Sakurada E, Ando A, Shimizu S, Ogawa J. 2013. Metabolic engineering for the production of polysaturated fatty acids by oleaginous fungus Mortierella alpina 15-4. J Biosci Bioeng 116:417–422. https://doi.org/10.1016/j.jbiosc.2013.04.008.

4. Han SF, Jin WB, Tu RJ, Wu WM. 2015. Biofuel production from microalgae as feedstock: current status and potential. Crit Rev Biotechnol 35:255–268. https://doi.org/10.3109/07388551.2013.835301.

5. Cho H, Cronan JE, Jr. 1995. Defective export of a periplasmic enzyme disrupts regulation of fatty acid synthase. J Biol Chem 270:4216–4219. https://doi.org/10.1074/jbc.270.9.4216.

6. Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R. 2011. Engineered re-

7. Lennen RM, P

8. Yan Q, P

9. Ikeda M, Miyamoto A, Mutoh S, Kitano Y, Tajima M, Shirakura D, Takasaki M, Urabayashi A, Mimura A, Muramatsu T, Mitsuhashi S, Ohnishi J, Hayashi M, Ikeda M. 2018. The FadR-type transcriptional regulator FasR of Corynebacterium glutamicum controls genes of lipid synthesis during growth on acetate. Mol Microbiol 78:253–265. https://doi.org/10.1111/mib.13555.

10. Takeno S, Takasaki M, Urabayashi A, Mitsuhashi S, Ohnishi J, Hayashi M, Ikeda M. 2004. A gene homologous to the national regulator FasR of Corynebacterium glutamicum for the production of polyunsaturated fatty acids by oleaginous fungus Mortierella alpina for the production of polyunsaturated fatty acids by oleaginous fungus Mortierella alpina. Appl Microbiol Biotechnol 63:592–601. https://doi.org/10.1007/s00253-003-1402-8.

11. Martin JF, Barreiro E, González-Lavado E, Barriuso M. 2003. Ribosomal
RNA and ribosomal proteins in corynebacteria. J Biotechnol 104:41–53. https://doi.org/10.1016/j.jbiotec.2003.08.003.

39. Ikeda M, Nakagawa S. 2003. The Corynebacterium glutamicum genome: features and impacts on biotechnological processes. Appl Microbiol Biotechnol 62:99–109. https://doi.org/10.1007/s00253-003-1328-1.

40. Takeno S, Ohnishi J, Komatsu T, Masaki T, Sen K, Ikeda M. 2007. Anaerobic growth and potential for amino acid production by nitrate respiration in Corynebacterium glutamicum. Appl Microbiol Biotechnol 75:1173–1182. https://doi.org/10.1007/s00253-007-0926-8.

41. Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

42. Ohnishi J, Mitsuhashi S, Hayashi M, Ando S, Yokoi H, Ochiai K, Ikeda M. 2002. A novel methodology employing Corynebacterium glutamicum genome information to generate a new L-lysine-producing mutant. Appl Microbiol Biotechnol 58:217–223. https://doi.org/10.1007/s00253-001-0883-6.

43. Krings E, Krumbach K, Bathe B, Kelle R, Wendisch VF, Sahm H, Eggeling L. 2006. Characterization of myo-inositol utilization by Corynebacterium glutamicum: the stimulon, identification of transporters, and influence on L-lysine formation. J Bacteriol 188:8054–8061. https://doi.org/10.1128/JB.00935-06.

44. Barnes EM, Jr. 1975. Long-chain fatty acyl thioesterase I and II from Escherichia coli. Methods Enzymol 35:102–109. https://doi.org/10.1016/0076-6879(75)35144-6.

45. Ichihara K, Shibasaki Y. 1991. An enzyme-coupled assay for acyl-CoA synthetase. J Lipid Res 32:1709–1712.

46. Hayashi M, Mizoguchi H, Shiraiishi N, Obayashi M, Nakagawa S, Imaj M, Watanabe S, Ota T, Ikeda M. 2002. Transcriptome analysis of acetate metabolism in Corynebacterium glutamicum using a newly developed metabolic array. Biosci Biotechnol Biochem 66:1337–1344. https://doi.org/10.1271/bbb.66.1337.

47. Kind S, Kreye S, Wittmann C. 2011. Metabolic engineering of cellular transport for overproduction of the platform chemical 1,5-diaminopentane in Corynebacterium glutamicum. Metab Eng 13:617–627. https://doi.org/10.1016/j.ymben.2011.07.006.

48. Katayama S, Kukita T, Ishikawa E, Nakashima S, Masuda S, Kanda T, Akiyama H, Teshima R, Nakamura S. 2013. Apple polyphenols suppress antigen presentation of ovalbumin by THP-1-derived dendritic cells. Food Chem 138:757–761. https://doi.org/10.1016/j.foodchem.2012.10.076.

49. Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108. https://doi.org/10.1038/nprot.2008.73.

50. Takeno S, Hori K, Ohtani S, Mimura A, Mitsuhashi S, Ikeda M. 2016. L-Lysine production independent of the oxidative pentose phosphate pathway by Corynebacterium glutamicum with the Streptococcus mutans gapN gene. Metab Eng 37:1–10. https://doi.org/10.1016/j.ymben.2016.03.007.

51. Kawaguchi A, Okuda S. 1977. Fatty acid synthetase from Brevibacterium ammoniagenes: formation of monounsaturated fatty acids by a multienzyme complex. Proc Natl Acad Sci U S A 74:3180–3183. https://doi.org/10.1073/pnas.74.8.3180.

52. Erffl JD. 1973. Acetyl-CoA and propionyl-CoA carboxylation by Mycobacterium phlei: partial purification and some properties of the enzyme. Biochim Biophys Acta 316:143–155. https://doi.org/10.1016/0005-2760(73)90004-0.

53. Morishima N, Ikai A. 1987. Active site organization of bacterial type I fatty acid synthetase. J Biochem 102:1451–1457. https://doi.org/10.1093/oxfordjournals.jbchem.a122191.

54. Irzik K, van Ooyen J, Gätgens J, Krumbach K, Bott M, Eggeling L. 2014. Acyl-CoA sensing by FasR to adjust fatty acid synthesis in Corynebacterium glutamicum. J Biotechnol 192:96–101. https://doi.org/10.1016/j.jbiotec.2014.10.031.