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Development of Fatty Acid-Producing *Corynebacterium glutamicum* Strains

Running title: Fatty Acid Production by *C. glutamicum*

Seiki Takeno,[a] Manami Takasaki,[a] Akinobu Urabayashi,[a] Akinori Mimura,[a] Tetsuhiro Muramatsu,[a] Satoshi Mitsuhashi,[b] and Masato Ikeda [a]*

Department of Bioscience and Biotechnology, Faculty of Agriculture
Shinshu University, Nagano 399-4598, Japan,[a] and
Bioprocess Development Center, Kyowa Hakko Bio Co., Ltd.,
Tsukuba, Ibaraki 305-0841, Japan[b]

***Corresponding author:** Mailing address: Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Nagano 399-4598, Japan
Phone: +81-265-77-1614. Fax: +81-265-77-1629. E-mail: m_ikeda@shinshu-u.ac.jp

ABSTRACT To date, no information has been made available on the genetic traits that lead to increased carbon flow into the fatty-acid-biosynthetic pathway in *Corynebacterium glutamicum*. To develop basic technologies for engineering, we employed an approach that begins by isolating a fatty acid-secreting mutant without depending on mutagenic treatment. This was followed by genome analysis to characterize the genetic background. The selection of spontaneous mutants resistant to the palmitic acid ester surfactant Tween 40 resulted in isolation of a desired mutant that produced oleic acid, suggesting that a single mutation would cause increased carbon flow down the pathway and subsequent excretion of the oversupplied fatty acid into the medium. Two additional rounds of the selection of spontaneous cerulenin-resistant mutants led to increased production of the fatty acid in a step-wise manner. Whole genome sequencing of the resulting best strain identified three specific mutations (*fasR20*, *fasA63*^{up}, and *fasA2623*). Allele-specific PCR analysis showed that the mutations have arisen in that order. Reconstitution experiments using these mutations revealed that only *fasR20* gave rise to oleic acid production in the wild strain. The other two mutations contributed to an increase in oleic acid production. Deletion of *fasR* in the wild strain led to oleic acid production as well. Reverse transcription-quantitative PCR analysis revealed that the *fasR20* mutation brought about upregulation of the *fasA* and *fasB* genes encoding fatty acid synthases IA and IB, respectively, by 1.31 ± 0.11-fold and 1.29 ± 0.12-fold, respectively, and of the *accD1* gene encoding the β-subunit of acetyl-CoA carboxylase by 3.56 ± 0.97-fold. On the other hand, the *fasA63*^{up} mutation upregulated the *fasA* gene by 2.67 ± 0.16-fold. In flask cultivation with 1% glucose, the *fasR20-fasA63*^{up}*-fasA2623* triple mutant produced approximately 280 mg of fatty acids per liter, which were comprised mainly of oleic acid (208

mg/liter) and palmitic acid (47 mg/liter).

INTRODUCTION

Lipids and their related compounds comprise a variety of useful materials, such as arachidonic, eicosapentaenoic, and docosahexaenoic acids that are functional lipids (1), prostaglandins and leukotrienes that are used as pharmaceuticals (2), biotin and α-lipoic acid that have pharmaceutical and cosmetic uses (3-5), and hydrocarbons and fatty acid ethyl esters that are used as fuels (6, 7). Since most of these compounds are derived via the fatty-acid-synthetic pathway, increasing carbon flow into this pathway is an important consideration in producing these compounds by the fermentation method.

Although there are numerous articles on lipid production by oleaginous fungi and yeasts (8, 9), attempts at using bacteria for that purpose remain limited (10-12). A pioneering study that showed the bacterial production of fatty acids using genetically engineered *E. coli* was performed by Cho and Cronan (11). They demonstrated that cytosolic expression of the periplasmic enzyme acyl-acyl carrier protein (ACP) thioesterase I (TesA) resulted in extracellular production of free fatty acids. This phenomenon has been reasonably explained by avoidance of the regulatory mechanism of fatty acid synthesis through the TesA-catalyzed cleavage of acyl-ACP, which acts as a feedback inhibitor of fatty-acid-synthetic enzymes acetyl-CoA carboxylase, FabH, and FabI (11). Most of the later studies on the bacterial production of fatty acids and their derivatives have been based on this technique (13, 14). Another representative work is the establishment of a reversal β-oxidation cycle in *E. coli*, which also led to extracellular production of free fatty acids (12). The advantage of this approach is that the engineered pathway directly uses acetyl-CoA instead of malonyl-CoA for acyl-chain

elongation, and can thus bypass the ATP-consuming step required for malonyl-CoA formation. Despite these positive results, fatty acid productivities remain far below a practical level. In addition, the bacterial production platform has exclusively depended on *E. coli*, except for one example of *cyanobacterium*, to which the *E. coli* TesA technique has been applied (13).

Our objective is to develop the basic technologies to produce fatty acids by using *Corynebacterium glutamicum*. This bacterium has long been used for the industrial production of a variety of amino acids including L-glutamic acid and L-lysine (15). It has also recently been developed as a production platform for various commodity chemicals (16, 17, 18), fuel alcohols (19, 20), carotenoids (21), and heterologous proteins (22). However, there are no reports of fatty acid production by this bacterium, except for undesired production of acetate, a water-soluble short-chain fatty acid, as a byproduct (23). To the best of our knowledge, no attempts have been made to improve carbon flow into the fatty-acid-biosynthetic pathway. In this context, it seems worthwhile to verify the feasibility of this bacterium as a potential workhorse for fatty acid production.

With respect to fatty acid biosynthesis in *C. glutamicum*, there are some genetic and functional studies on the relevant genes (24-28). Unlike the majority of bacteria including *E. coli* and *B subtilis*, coryneform bacteria, such as genera *Corynebacterium* and *Mycobacterium*, are known to possess type I fatty acid synthase (Fas) (29), a multienzyme that performs successive cycles of fatty acid synthesis, into which all activities required for fatty acid elongation are integrated (29). In addition, *Corynebacterium* fatty acid synthesis is thought to differ from that of common bacteria in that the donor of two-carbon units and the end product are CoA derivatives instead of

ACP derivatives. This was demonstrated using the purified Fas from *Corynebacterium ammoniagenes* (30), which is closely related to *C. glutamicum*. With regard to the regulatory mechanism of fatty acid biosynthesis, the details are not fully understood. It was only recently shown that the relevant biosynthesis genes were transcriptionally regulated by the TetR-type transcriptional regulator FasR (28). Fatty acid metabolism and its predicted regulatory mechanism in *C. glutamicum* are shown in Fig. 1.

In this study, we initially investigated whether a desired fatty acid-producing mutant can be obtained from wild-type *C. glutamicum*. Our strategies were (i) to isolate a mutant that secretes oleic acid, a major fatty acid in the *C. glutamicum* membrane lipid (27), as an index of fatty acid production, and (ii) to identify the causal mutations through genome analysis. For this purpose, we attempted to induce mutants that acquired desired phenotypes without using mutagenic treatment. Compared to the conventional mutagenic procedure that depends on chemical mutagens or ultraviolet, the selection of a desired phenotype by spontaneous mutation is undoubtedly less efficient, but seems to permit the accumulation of a minimum number of beneficial mutations even if the process is repeated. If this is true, genome analysis can be expected to directly decipher the results leading to desired phenotypes, and thereby define the genetic background that is required to achieve the production. Described herein is the first demonstration of such strain development undertaken toward fatty acid production by *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and chemicals. The wild-type *C. glutamicum* strain ATCC 13032 was used in this study. *C. glutamicum* OLA-15, which was used as

an indicator strain for agar piece assays, is an oleic acid-auxotrophic mutant derived by a round of mutagenesis from the wild strain. *Escherichia coli* DH5α was used as a host for DNA manipulation. The plasmid pCS299P (31), a *C. glutamicum-E. coli* shuttle vector, was used to clone the PCR products. The plasmid pESB30 (31), which is nonreplicative in *C. glutamicum*, is a vector for gene replacement in *C. glutamicum*. The primer sequences used in this study are listed in Table S1. All primers were designed based on the genomic sequence of *C. glutamicum* ATCC 13032 (BA000036), which is publicly available at http://www.genome.jp/kegg/genes.html (32). The chemical compounds Tween 40 and cerulenin were purchased from Nakalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd (Osaka, Japan), respectively.

Media and culture conditions. Complete medium BY (33) and minimal medium MM (33) were used for the cultivation of wild-type ATCC 13032 and its derivatives. MM medium contained 1% glucose as the sole carbon source. Solid plates were made by the addition of Bacto agar (Difco) to 1.5%. For lipid production in liquid culture, a 3-ml sample of the seed culture grown in BY medium to the mid-exponential phase at 30ºC was inoculated into a 300-ml baffled Erlenmeyer flask containing 30 ml of MM medium, followed by cultivation at 30ºC using a rotary shaker at 200 rpm.

Agar piece assays for oleic acid production. Microbiological assay for oleic acid was performed with an agar piece method, essentially as described previously (34).

Recombinant DNA techniques. Standard protocols (35) were used for the construction, purification, and analysis of plasmid DNA and for the transformation of *E. coli*. The

144 extraction of *C. glutamicum* chromosomal DNA and transformation of *C. glutamicum*
145 by electroporation were carried out as described previously (33).
146
147 **Identification of mutations in fatty acid-producing mutants.** Mutations in strain
148 PCC-6 were identified via a comparative genome analysis using the wild-type ATCC
149 13032 genome as a reference (http://www.genome.jp/kegg/genes.html). Whole genome
150 sequencing of strain PCC-6 was conducted by Takara Bio Inc (Shiga, Japan) using
151 Illumina Genome Analyzer IIx (Illumina, San Diego, CA). In regard to the three specific
152 mutations found in strain PCC-6, allele-specific PCR (36) was conducted to examine
153 the presence or absence of each specific mutation in strains PAS-15 and PC-33.
154
155 **Introduction of specific mutations into the genome.** Plasmids pCfasR20, pCfasA63^{up},
156 and pCfasA2623, which were used for the introduction of specific mutations into the *C.*
157 *glutamicum* genome, were constructed as follows. The mutated *fasR* gene region was
158 PCR amplified using primers Cgl2490up700F and Cgl2490down500RFbaI with the
159 genomic DNA from strain PCC-6 as a template, producing the 1.3-kb fragment. On the
160 other hand, a region upstream of the *fasA* gene of strain PCC-6 was amplified using
161 Cgl0836up900FFbaI and Cgl0836inn700RFbaI, producing the 1.7-kb fragment.
162 Similarly, the mutated *fasA* gene region was amplified using primers
163 Cgl0836inn700FFbaI and Cgl0836down200RFbaI with the genomic DNA of strain
164 PCC-6, producing the 2.1-kb fragment. After verification by DNA sequencing, each
165 PCR fragment that contained the corresponding point mutation in its midportion was
166 digested with BclI and then ligated to BamHI-digested pESB30 to yield the intended
167 plasmid. The introduction of each specific mutation into the *C. glutamicum* genome was

accomplished using the corresponding plasmid via two recombination events, as described previously (37). The presence of the mutation(s) was confirmed by allele-specific PCR and DNA sequencing.

Chromosomal deletion of the *fasR* gene. Plasmid pCΔfasR containing the internally deleted *fasR* gene was constructed as follows. The 5' region of the *fasR* gene was amplified using primers fasRup600FBglII and fasRFusR with wild-type ATCC 13032 genomic DNA as a template. Similarly, the 3' region of the gene was amplified using the primers fasRFusF and fasRdown800RBglII. The 5' and 3' regions were fused by PCR using the primers fasRup600FBglII and fasRdown800RBglII. The resulting 1.6-kb fragment containing the deleted *fasR* gene, which was shortened by an in-frame deletion from 639 to 60 bp, was digested with BglII and then ligated to BamHI-digested pESB30 to yield pCΔfasR. Defined chromosomal deletion of the *fasR* gene was accomplished via two recombination events using the plasmid.

RNA extraction, cDNA synthesis, and quantitative PCR. Extraction of total RNAs from *C. glutamicum* strains and subsequent purification were performed according to the methods described previously (38). Synthesis of cDNA was performed using 300 ng of RNA by the methods described by Kind et al (17). Quantitative-PCR analysis was performed by the method described by Katayama et al (39). The gene expression levels were standardized to the constitutive expression level of 16S rRNA, and calculated using the comparative C_T method (40).

Quantitative determination of lipids. Total lipids were extracted from culture

supernatant by the Bligh-Dyer method (41). The culture supernatant was prepared by removing cells through centrifugation at 10,000×g for 20 min and subsequent filtration with a Millex-MA filtrate unit (0.45 μm pore size; Millipore Corporation, Billerica, MA). The extracted total lipids were dissolved in 2 ml of chloroform (hereafter, the solution is referred to as extract A). Quantitative determination of lipids was conducted by Toray Research Center (Kanagawa, Japan) using gas chromatography and thin layer chromatography (TLC) as follows.

For free fatty acid analysis, 1 ml of extract A was evaporated under a nitrogen stream, suspended in a solvent containing 0.5 ml of benzene, 0.2 ml of methanol, and 1 ml of trimethylsilyldiazomethane, and then incubated at 60ºC for 1 hour for methyl-esterification of the free fatty acids. After the reaction, the mixture was evaporated under a nitrogen stream, dissolved in 1.0 ml of chloroform containing 0.005% of methyl heneicosanoate as an internal standard, and applied to a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and an Omegawax 320 column (Sigma-Aldrich, St. Louis, MO). The column temperature was kept at 50ºC for 1 min and then ramped to 270ºC at a rate of 8ºC/min. The injector and detector temperatures were held at 250ºC and 270ºC, respectively. Fatty acids were identified and quantified using authentic fatty acid methyl ester standards.

For phospholipid analysis, 1 ml of extract A was evaporated under a nitrogen stream, dissolved in 0.1 ml of chloroform, and applied onto HPTLC plates Silica gel 60 (Merck, Darmstadt, Germany). The solvent was chloroform/methanol/acetic acid/water, 125:75:6.5:5 (vol/vol/vol/vol). After separation, the plate was sprayed with 10% copper sulfate in 8% phosphoric acid solution, and baked for 30 min at 150ºC. The position of each lipid species was identified by comparison with the corresponding standard

supplied from Doosan Serdar Research Laboratories (Toronto, Ontario, Canada). The intensities of each spot were measured using an Image Master 1D Elite ver. 3.00 (Amersham Bioscience, Tokyo, Japan). Lipid species were quantified using the standard curves for each lipid drawn with serial dilutions of the standard substance.

Analysis. Bacterial growth was monitored by measuring the optical density at 660 nm (OD_{660}) of the culture broth with a Miniphoto 518R spectrophotometer (Taitec, Saitama, Japan). Glucose concentration was determined using Determinar GL-E (Kyowa Medex, Japan).

RESULTS

Screening of compounds to induce oleic acid-producing mutants. A chemical substance that satisfies the following criteria is assumed to be a specific inhibitor of fatty acid biosynthesis in *C. glutamicum*. As such, mutants resistant to the compound are likely to overproduce oleic acid, a major component of *C. glutamicum* membrane lipid (27): (i) *C. glutamicum* cells are subject to growth inhibition in the presence of the compound, and (ii) the growth inhibition is restored by the co-presence of oleic acid. After screening a variety of chemical substances, including known inhibitors of bacterial fatty acid biosynthesis (42), for such compounds, we found that the palmitic acid ester surfactant Tween 40, as well as the antibiotic cerulenin, satisfied the above criteria. Both the compounds have been suggested to have targets on fatty acid biosynthesis in the coryneform bacteria: the presence of Tween 40 in the culture caused a decreased amount of ACC β-subunit in *C. glutamicum* ATCC 13869 (24), whereas cerulenin inhibited fatty acid synthase from *C. ammoniagenes in vitro* (43). Both the

compounds have also been reported to trigger L-glutamate production by *C. glutamicum*, presumably by destabilization of membrane (44, 45).

Selection of spontaneous mutants resistant to Tween 40. Although both the compounds met our criteria, the phenotype of growth recovery by oleic acid was more prominent when Tween 40 was used. Thus, we first attempted to isolate spontaneous Tween 40-resistant mutants from wild-type *C. glutamicum* ATCC 13032. For this purpose, appropriate dilutions (10^{5-6} cells/ml) of the culture were spread on the MM agar plates containing the minimum inhibitory concentration (MIC) of Tween 40 (approximately 1.5 g/liter), and colonies that emerged on the plates after a 5-day cultivation were isolated. These Tween 40-resistant colonies were obtained at a frequency of approximately 10^{-4}. These resistant colonies were then examined for their abilities to produce oleic acid by agar piece assay using the oleic acid auxotroph OLA-15 as an indicator strain. As a result, more than half of the mutants examined were found to produce oleic acid whereas the wild strain never produced the fatty acid. Among these, the strain that gave the largest halo of the indicator strain was designated strain PAS-15 (Fig. 2). It was used as the parent strain to induce a second mutation.

Repeated selection of spontaneous cerulenin-resistant mutants. Since strain PAS-15 no longer exhibited sensitivity to Tween 40, even at 20 g per liter, we attempted to isolate spontaneous mutants resistant to the other compound, cerulenin, from the strain in the same way as when selecting Tween 40-resistant mutants. After cultivation for several days, colonies emerged on the MM agar plates containing the MIC (approximately 7.5 mg/liter) of cerulenin at a frequency of approximately 10^{-4}. These

resistant colonies were examined for their production of oleic acid by agar piece assay, revealing that approximately 5% of colonies showed a higher production of the fatty acid than the parental strain PAS-15. Among these, the strain that showed the highest production was designated strain PC-33 (Fig. 2). It was used as the parent strain to induce a third mutation. Because the strain still showed sensitivity to a higher concentration of cerulenin, we further induced higher resistance to cerulenin from the strain. When the spontaneous selection was conducted at the MIC (approximately 15 mg/liter) for strain PC-33, colonies emerged at a frequency of approximately 10^{-4}. Agar piece assay revealed that approximately 10% of colonies showed a higher production of the fatty acid than the parental strain PC-33. Among these, we selected the best producer, which was designated PCC-6 (Fig. 2).

Identification of mutations in strains PAS-15, PC-33, and PCC-6. Since the obtained strain PCC-6 had acquired the ability to produce a relatively large halo, for which we estimated the oleic acid level to be between 100 and 300 mg per liter, in our agar piece assay, we considered it worthwhile to analyze its genetic traits that were related to fatty acid production. To identify them, we conducted whole-genome sequencing of the strain, which revealed only three specific mutations (Fig. 3): a G-to-A exchange at nucleotide position 59 in the *fasR* gene, which led to the replacement of Ser-20 by Asn (designated mutation *fasR20*); a C-to-G exchange at 63 bp upstream of the *fasA* gene (designated mutation *fasA63*^{up}); and a C-to-T exchange at nucleotide position 7868 in the *fasA* gene, which led to the replacement of Ala-2623 by Val (designated mutation *fasA2623*). Since the *fasR* and *fasA* genes are known to encode the transcriptional regulator FasR and the fatty acid synthase FasA, respectively (27, 28),

the three mutations identified were all suggested to be related to fatty acid biosynthesis. Subsequent allele-specific PCR revealed that the initially obtained strain PAS-15 carried the *fasR20* mutation whereas the next strain PC-33 carried the *fasA63*^{up} mutation, in addition to *fasR20*, indicating that the mutations arose in the following order: *fasR20*, *fasA63*^{up}, and *fasA2623* (Fig. 3). This also suggests that the *fasR20* mutation is responsible for the Tween 40 resistance, whereas the *fasA63*^{up} and *fasA2623* mutations are responsible for resistance to the lower and higher concentrations of cerulenin, respectively.

Reconstitutions of defined mutations in a wild-type genome and their effects on oleic acid production. To examine the relevance of the three mutations to oleic acid production, we first introduced them into the wild-type genome separately and examined their abilities to produce oleic acid (Fig. 4). Agar piece assay showed that only *fasR20* gave rise to oleic acid production in the wild strain, whereas the other two mutations showed no significant effect on production. We also examined the effect of the in-frame deletion of the *fasR* inner sequence (designated Δ*fasR*) on production in the wild strain, which revealed that the modification resulted in almost the same level of oleic acid production as in the case of *fasR20* (Fig. 4). Next, we examined the effects of the combination of *fasR20* with either *fasA63*^{up} or *fasA2623* on production (Fig. 4). When *fasR20* was combined with *fasA63*^{up} in the wild-type genome, increased oleic acid production was observed, compared with that obtained with *fasR20* alone. The combination of *fasR20* and *fasA2623* resulted in an oleic acid production level that was comparable to that obtained with *fasR20* alone. On the other hand, the combination of *fasA*63^{up} and *fasA2623* in the wild-type genome resulted in no oleic acid production.

When all three mutations were combined in the wild-type genome, the highest oleic acid production was observed among all combinations, as expected (Fig. 4). These results indicate that the loss of the function of *fasR* is of primary importance for fatty acid production by *C. glutamicum*, and that the *fasA63*^{up} and *fasA2623* mutations positively affect carbon flow down the pathway. The *fasA2623* mutation seemed to be effective, especially under the background of *fasR20* and *fasA63*^{up}.

Effect of the *fasR20* and *fasA63*^{up} mutations on the transcript levels of the fatty acid biosynthesis genes. Apart from the *fasA2623* mutation that was thought to affect the enzymatic properties of FasA (see Discussion), the *fasR20* and *fasA63*^{up} mutations were both considered to affect the transcript levels of the relevant genes, because the former is a missense mutation within the transcriptional regulator FasR and the latter is located near the predicted promoter-operator regions of the *fasA* gene (Fig. 3). Accordingly, we used reverse transcription-quantitative PCR (RT-qPCR) to investigate the transcript levels of the fatty acid biosynthesis genes *fasA, fasB, accD1*, and *accBC* in the strains carrying the two mutations individually or in combination. As shown in Fig. 5, the *fasR20* mutation increased the transcript levels of *accD1* by 3.56 ± 0.97-fold, as well as both *fasA* and *fasB* by 1.31 ± 0.11-fold and 1.29 ± 0.12-fold, respectively, whereas the mutation had little influence on *accBC* gene expression. Similar behavior in the transcript levels was observed in the Δ*fasR* strain (Fig. 5). On the other hand, the *fasA63*^{up} mutation led to 2.67 ± 0.16-fold increase in the transcript level of *fasA*. The presence of both the *fasR20* and *fasA63*^{up} mutations resulted in an additive effect on *fasA* gene expression.

Lipid production by strain PCC-6. Although strain PCC-6 produced oleic acid from glucose, we needed to determine what kinds of lipids were produced and what their yields were. To clarify this, strain PCC-6, as well as wild-type ATCC 13032, was aerobically cultivated in 30 ml of MM medium containing 1% glucose in a 300-ml baffled Erlenmeyer flask (Fig. 6). Under these conditions, strain PCC-6 showed a lower growth rate and a lower final OD_{660} than the wild strain, probably due to production of fatty acids and their negative effects on cell physiology (46). After glucose was consumed, the cells were removed by centrifugation followed by filtration and the culture supernatant was subject to lipid analysis. As shown in Table 1, wild-type ATCC 13032 produced only a trace amount of lipids. In contrast, strain PCC-6 produced 279.95 ± 8.50 mg of free fatty acids and 43.18 ± 1.84 mg of phospholipids per liter. The fatty acids consisted mainly of oleic acid (208.10 ± 5.67 mg/liter) and palmitic acid (46.93 ± 2.03 mg/liter), both accounting for 91.10% of the total free fatty acids produced in the culture supernatant. The conversion yield of the total fatty acids on glucose was 2.80 ± 0.09% (wt/wt). Since the theoretical yield of oleic acid on glucose is estimated to be 34.8% (wt/wt) on the basis of our calculation, the production level of strain PCC-6 is considered to be less than 10% of the theoretical yield.

DISCUSSION

Despite a broad product portfolio for *C. glutamicum* (15, 17, 18, 19, 21), lipids and their related compounds have not been intensively developed for production. In this study, we demonstrated for the first time that this organism has the capability of producing considerable amounts of fatty acids directly from sugar, thus expanding its product portfolio to lipids. This raises the possibility of developing production processes by *C.*

glutamicum not only for fatty acids but also for other useful compounds that are derived via the fatty-acid-biosynthetic pathway. To date, no information is available on what kind of modifications or selections contribute to increased carbon flow into the fatty-acid-biosynthetic pathway in this organism. This study is the first to report not only the selection methods but also the genetic traits that cause fatty acid production.

The three specific mutations, *fasR20*, *fasA63*^{up}, and *fasA2623*, identified as genetic traits that are useful for fatty acid production are all related to fatty acid biosynthesis, and no mutation that is related to fatty acid transport is included. This suggests that deregulation of the fatty-acid-biosynthetic pathway would cause carbon flow down the pathway and that the oversupplied acyl-CoAs would be excreted into the medium as free fatty acids without undergoing degradation in this organism. The latter hypothesis is supported by the *C. glutamicum* genome information, which shows a lack of some of the genes responsible for the β-oxidation of fatty acids (Fig. 1) (47). In fact, unlike *E. coli*, wild-type *C. glutamicum* hardly grew on MM medium containing 10 g of oleic acid per liter as the sole carbon source (data not shown). The relevance of each mutation to fatty acid production is discussed below.

The *fasR20* mutation conferred oleic acid production on wild-type *C. glutamicum*, concomitantly with the Tween 40-resistant phenotype (Figs. 2 and 4). Since this mutation more or less increased the expression levels of *accD1*, *fasA*, and *fasB* (Fig. 5), the effect of the mutation on the production is reasonably explained by derepression of the key regulatory genes in the fatty-acid-biosynthetic pathway. Considering that the *fasR* gene product is thought to be a repressor protein for fatty acid biosynthesis (28) and also that deletion of the gene in the wild strain caused similar oleic acid production (Fig. 4), the *fasR20* mutation would cause the functional

384 impairment of the repressor protein. In this context, it has been suggested that the FasR
385 protein, combined with the effector acyl-CoA, binds to *fasO* sites upstream of the
386 corresponding genes, and thereby suppresses their expression (28). Based on this
387 mechanism, the *fasR20* mutation is likely to interfere with the formation of the
388 FasR-acyl-CoA complex or binding of the complex to the *fasO* sites. Taken all together,
389 the reason why the Tween 40-resistant phenotype resulted in oleic acid production can
390 be explained as follows. In the wild strain, the palmitic acid ester surfactant Tween 40
391 probably triggers the FasR-mediated repression of fatty acid biosynthesis, which causes
392 deprivation of essential lipids and results in growth inhibition. However, this Tween
393 40-triggered repression mechanism can be bypassed in the *fasR*-defective mutant, thus
394 leading to the Tween 40-resistant phenotype, accompanied by derepression of fatty acid
395 biosynthesis and subsequent oleic acid production. This speculation is supported by our
396 findings that the growth inhibitory effect of Tween 40 on wild-type *C. glutamicum* is
397 restored either by the co-presence of oleic acid or by the loss of the function of *fasR*
398 (data not shown).

399 The *fasA63*^{up} mutation, which is located upstream of the *fasA* coding region,
400 was obtained by the selection of a relatively low concentration of cerulenin under the
401 genetic background of *fasR20*. Since the mutation significantly increased the transcript
402 level of the *fasA* gene (Fig. 5), the effect of the mutation on oleic production is
403 comprehensible by an increased amount of the FasA enzyme that is responsible for oleic
404 acid synthesis (27, 48). Considering that cerulenin is known to inhibit Fas from the
405 closely related *C. ammoniagenes* (43), as well as *E. coli* FabF and FabB (49, 50), it is
406 reasonable to assume that the agent also inhibits *C. glutamicum* FasA, which causes
407 deprivation of essential lipids and results in growth inhibition. This hypothesis is

consistent with the previous observation that inactivation of FasA in *C. glutamicum* resulted in no growth in MM medium and that this growth impairment was recovered by oleic acid supplementation (27). Presumably, the mutants with increased transcript levels of *fasA* could overcome the cerulenin-caused inhibition of FasA through the dosage effect of the FasA molecules. This explains why the cerulenin-resistant phenotype was caused by the mutation and resulted in increased oleic acid production. Although the *fasA63*^{up} mutation is located outside of the putative promoter-operator regions of the *fasA* gene (Fig. 3), our RT-qPCR data suggest that the mutation site is undoubtedly involved in the *fasA* gene expression.

The *fasA2623* mutation, which is present in the *fasA* coding region, was obtained by the selection of a relatively high concentration of cerulenin under the genetic background of *fasR20* and *fasA63*^{up}. The mutation is present within a motif sequence (PROSITE motif PS00606) for a 3-ketoacyl-ACP synthase (KS) active site in the deduced amino acid sequence of FasA. In this regard, the *E. coli* KS enzyme FabH, which has the same motif sequence (http://ccdb.wishartlab.com/CCDB/cgi-bin/ECARD_HTML_NEW.cgi?ECARD=FABH_ECOLI.ecard), has been reported to be feedback-inhibited by long-chain (12-20 carbons) acyl-ACPs through a mixed type of inhibition, namely, a combination of competitive and noncompetitive inhibition with respect to acetyl-CoA (51). If *C. glutamicum* FasA is regulated at its KS domain in the same manner as seen for *E. coli* FabH, it seems reasonable to speculate that the *fasA2623* mutation alleviates the feedback inhibition and thereby results in increased oleic acid production. In *E. coli*, cerulenin is known to inhibit KS by covalently binding to the active-center cysteine (49). This cysteine residue is assumed to correspond to Cys2619 of the deduced amino acid

sequence of *C. glutamicum* FasA, based on sequence alignment. Taking this into consideration, it is likely that the *fasA2623* mutation, which is located very near to the predicted active center and gives rise to a change from alanine to valine with a longer side chain, may cause steric hindrance to the binding of cerulenin, thereby resulting in cerulenin resistance. This may also be the mechanism of the possible relief of the mutated FasA enzyme from the feedback inhibition.

The reconstitution experiments of three specific mutations under the wild-type background (Fig. 4) have demonstrated that the *fasR* mutation is of primary importance for fatty acid production by *C. glutamicum*. To confirm this, we sequenced the *fasR* genes from an additional 30 oleic acid-producing mutants selected by Tween 40 resistance and found that all *fasR* genes carried mutations, including single-base substitutions (10 cases of 30 mutants), single-base insertions (3 cases), a 165-bp deletion (1 case), and insertion of IS*Cg1a* (15 cases) or IS*Cg13b* (1 case) (data not shown). These results strongly suggest that the loss of the function of *fasR* is essential for fatty acid production by *C. glutamicum*. To date, it has not been reported that inactivation of *fasR* induces fatty acid production in *C. glutamicum*, despite the study on the *fasR* gene (28).

As described in the Introduction section, *E. coli* has recently been used for studying fatty acid production. Since the first report on fatty acid production by *E. coli* overexpressing the modified acyl-ACP thioesterase gene ‘*tesA* (11), overexpression of the enzyme has become a common strategy for fatty acid production by *E. coli*. A basic concept for the production is avoidance of the regulatory mechanism of fatty acid synthesis through the thioesterase-catalyzed cleavage of acyl-ACP. On the other hand, in our case with *C. glutamicum*, the defined genetic modifications to fatty acid

biosynthesis resulted in fatty acid production without modification to the acyl-ACP thioesterase enzyme. This raises a question of how the oversupplied acyl-CoAs, end products of the fatty acid biosynthesis in this organism, would be excreted into the medium as free fatty acids. In regard to this, we found that *C. glutamicum* originally had a high level of thioesterase activity (1.27 ± 0.018 units/mg of protein) in the soluble fraction prepared from cells grown in MM medium. This activity is of a level comparable to that obtained from a '*tesA*-overexpressing *E. coli* (1.29 ± 0.11 units/mg of protein) and is of an approximately 16-fold higher level compared with that from a non-'*tesA*-overexpressing *E. coli*. Taking this into consideration, *C. glutamicum* is likely to possess a specific mechanism for maintaining lipid homeostasis even in the presence of high thioesterase activity. The *C. glutamicum* genome indicates the presence of three putative acyl-CoA thioesterases (Cgl0091, Cgl1664, and Cgl2451). The involvement of these genes in fatty acid production, along with the mechanism of free fatty acid secretion, needs to be clarified in a future study.

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REFERENCES

1. **Horrobin DF.** 1992. Nutritional and medical importance of gamma-linolenic acid. Prog. Lipid Res. **31:**163-194.

2. **Smith WL, Borgeat P.** 1985. The eicosanoids: prostaglandins, thromboxanes, leukotrienes, and hydroxy-eicosaenoic acids, p. 325-360. *In* Vance DE, Vance JE (ed), Biochemistry of lipids and membranes. Benjamin/Cummings, Menlo Park, CA.
3. **Bilska A, Włodek L.** 2005. Lipoic acid—the drug of the future? Pharmacol. Rep. **57:**570-577.
4. **Packer L, Witt EH, Tritschler HJ.** 1995. Alpha-lipoic acid as a biological antioxidant. Free Radic. Biol. Med. **19:**227-250.
5. **Streit WR, Entcheva P.** 2003. Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production. Appl. Microbiol. Biotechnol. **61:**21-31.
6. **Kalscheuer R, Stölting T, Steinbüchel A.** 2006. Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology **152:**2529-2536.
7. **Metzger P, Largeau C.** 2005. *Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids. Appl. Microbiol. Biotechnol. **66:**486-496.
8. **Sakuradani E, Ando A. Shimizu S. Ogawa J.** 2013. Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S-4. J. Biosci. Bioeng., in press. doi: 10.1016/j.jbiosc.2013.04.008.
9. **Beopoulos A, Nicaud JM, Gaillardin C.** 2011. An overview of lipid metabolism in yeasts and its impact on biological processes. Appl. Microbiol. Biotechnol. **90:**1193-1206.
10. **Lennen RM, Pfleger BF.** 2012. Engineering *Escherichia coli* to synthesize free fatty acids. Trends Biotechnol. **30:**659-667.
11. **Cho H, Cronan JE, Jr.** 1995. Defective export of a periplasmic enzyme disrupts

regulation of fatty acid synthesis. J. Biol. Chem. **270:**4216-4219.

12. **Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R.** 2011. Engineered reversal of the β-oxidation cycle for the synthesis of fuels and chemicals. Nature **476:**355-359.
13. **Liu X, Brune D, Vermaas W, Curtiss R, III.** 2011. Production and secretion of fatty acids in genetically engineered cyanobacteria. Proc. Natl. Acad. Sci. U. S. A. **108:**6899-6904.
14. **Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, del Cardayre SB, Keasling JD.** 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature **463:**559-562.
15. **Ikeda M, Takeno S.** 2013. Amino acid production by *Corynebacterium glutamicum*, p 107-147. *In* Yukawa H, Inui M, (ed), *Corynebacterium glutamicum.* Microbiology monographs 23. Springer, Berlin, Germany.
16. **Okino S, Noburyu R, Suda M, Jojima T, Inui M, Yukawa H.** 2008. An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Appl. Microbiol. Biotechnol. **81:**459-464.
17. **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.
18. **Song Y, Matsumoto K, Tanaka T, Kondo A, Taguchi S.** 2013. Single-step production of polyhydroxybutyrate from starch by using α-amylase cell-surface displaying system of *Corynebacterium glutamicum*. J. Biosci. Bioeng. **115:**12-14.
19. **Inui M, Kawaguchi H, Murakami S, Vertès AA, Yukawa H.** 2004. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under

528 oxygen-deprivation conditions. J. Mol. Microbiol. Biotechnol. **8:**243-254.
529 20. **Blombach B, Riester T, Wieschalka S, Ziert C, Youn JW, Wendisch VF,**
530 **Eikmanns BJ.** 2004. *Corynebacterium glutamicum* tailored for efficient isobutanol
531 production. Appl. Environ. Microbiol. **77:**3300-3310.
532 21. **Heider SAE, Peters-Wendisch P, Wendisch VF.** 2012. Carotenoid biosynthesis
533 and overproduction in *Corynebacterium glutamicum*. BMC Microbiol. **12:**198-208.
534 22. **Kikuchi Y, Itaya H, Date M, Matsui K, Wu LF.** 2009. TatABC overexpression
535 improves *Corynebacterium glutamicum* Tat-dependent protein secretion. Appl.
536 Environ. Microbiol. **75:**603-607.
537 23. **Inui M, Murakami S, Okino S, Kawaguchi H, Vertès AA, Yukawa H.** 2004.
538 Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate
539 productions under oxygen deprivation conditions. J. Mol. Microbiol. Biotechnol.
540 **7:**182-196.
541 24. **Kimura E, Yagoshi C, Kawahara Y, Ohsumi T, Nakamatsu T, Tokuda H.** 1999.
542 Glutamate overproduction in *Corynebacterium glutamicum* triggered by a decrease
543 in the level of a complex comprising DtsR and a biotin-containing subunit. Biosci.
544 Biotechnol. Biochem. **63:**1274-1278.
545 25. **Gande R, Gibson KJC, Brown AK, Krumbach K, Dover LG, Sahm H,**
546 **Shioyama S, Oikawa T, Besra GS, Eggeling L.** 2004. Acyl-CoA carboxylase
547 (*accD2* and *accD3*), together with a unique polyketide synthase (*Cg-pks*), are key to
548 mycolic acid biosynthesis in *Corynebacterianeae* such as *Corynebacterium*
549 *glutamicum* and *Mycobacterium tuberculosis*. J. Biol. Chem. **279:** 44847-44857.
550 26. **Jäger W, Peters-Wendisch PG, Kalinowski J, Pühler A.** 1996. A
551 *Corynebacterium glutamicum* gene encoding a two-domain protein similar to biotin

carboxylases and biotin-carboxyl-carrier protein. Arch. Microbiol. **166:**76-82.

27. **Radmacher E, Alderwick LJ, Besra GS, Brown AK, K, Gibson KJC, Sahm H, Eggeling L.** 2005. Two functional FAS-I type fatty acid synthases in *Corynebacterium glutamicum*. Microbiology **151:**2421-2427.
28. **Nickel J, Irzik K, van Ooyen J, Eggeling L.** 2010. The TetR-type transcriptional regulator FasR of *Corynebacterium glutamicum* controls genes of lipid synthesis during growth on acetate. Mol. Microbiol. **78:**253-265.
29. **Schweizer E, Hofmann J.** 2004. Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. Microbiol. Mol. Biol. Rev. **68:**501-517.
30. **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.
31. **Mitsuhashi S, Ohnishi J, Hayashi M, Ikeda M.** 2004. A gene homologous to β-type carbonic anhydrase is essential for the growth of *Corynebacterium glutamicum* under atmospheric conditions. Appl. Microbiol. Biotechnol. **63:**592-601.
32. **Ikeda M, Nakagawa S.** 2003. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. **62:** 99-109.
33. **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.
34. **Ikeda M, Miyamoto A, Mutoh S, Kitano Y, Tajima M, Shirakura D, Takasaki**

M, Mitsuhashi S, Takeno S. 2013. Development of biotin-prototrophic and -hyperauxotrophic *Corynebacterium glutamicum* strains. Appl. Environ. Microbiol. **79:**4586-4594.

35. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
36. **Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF.** 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. **17:**2503-2516.
37. **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.
38. **Hayashi M, Mizoguchi H, Shiraishi N, Obayashi M, Nakagawa S, Imai J, 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.
39. **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.
40. **Schmittgen TD, Livak K.** 2008. Analyzing real-time PCR data by the comparative C_T method. Nat. Protoc. **3:**1101-1108.
41. **Bligh EG, Dyer WJ.** 1959. A rapid method of total lipid extraction and purification.

Can. J. Biochem. Physiol. **37:**911-917.

42. **Heath RJ, White SW, Rock CO.** 2002. Inhibitors of fatty acid synthesis as antimicrobial chemotherapeutics. Appl. Microbiol. Biotechnol. **58:**695-703.
43. **Kawaguchi A, Tomada H, Okuda S, Awaya J, Omura S.** 1979. Cerulenin resistance in a cerulenin-producing fungus: isolation of cerulenin insensitive fatty acid synthetase. Arch. Biochem. Biophys. **197:**30-35.
44. **Asakura Y, Kimura E, Usuda Y, Kawahara Y, Matsui K, Osumi T, Nakamatsu T.** 2007. Altered metabolic flux due to deletion of *odhA* causes L-glutamate overproduction in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **73:**1308-1319.
45. **Hoischen C, Krämer R.** 1990. Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum*. J. Bacteriol. **172:**3409-3416.
46. **Desbois AP, Smith VJ.** 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl. Microbiol. Biotechnol. **85:**1629-1642.
47. **Barzantny H, Brune I, Tauch A.** 2012. Molecular basis of human body odour formation: insights deduced from corynebacterial genome sequences. Int. J. Cosmet. Sci. **34**:2-11.
48. **Stuible HP, Wagner C, Andreou I, Huter G, Haselmann J, Schweizer E.** 1996. Identification and functional differentiation of two type I fatty acid synthases in *Brevibacterium ammoniagenes*. J. Bacteriol. **178:**4787-4793.
49. **Magnuson K, Jackowski S, Rock CO, Cronan JE, Jr.** 1993. Regulation of fatty acid biosynthesis in *Escherichia coli*. Microbiol. Rev. **57:**522-542.

50. **Val D, Banu G, Seshadri K, Lindqvist Y, Dehesh K.** 2000. Re-engineering ketoacyl synthase specificity. Structure **8:**565-566.

51. **Heath RJ, Rock CO.** 1996. Inhibition of β-ketoacyl-acyl carrier protein synthase III (FabH) by acyl-acyl carrier protein in *Escherichia coli*. J. Biol. Chem. **271:**10996-11000.

52. **Erfle 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.

53. **Morishima N, Ikai A.** 1987. Active site organization of bacterial type I fatty acid synthetase. J. Biochem. **102:**1451-1457.

635 **FIGURE LEGENDS**

636 **FIG 1** Fatty acid metabolism and its predicted regulatory mechanism in *C. glutamicum*.
637 In coryneform bacteria, fatty acids are believed to be synthesized as acyl-CoAs (30),
638 which are destined for incorporation into the membrane phospholipid and the outer
639 layer component mycolic acid. Three genes responsible for the β-oxidation of fatty
640 acids are missing in the *C. glutamicum* genome (gray arrows) (47). The Tes enzyme is
641 assumed to be involved in the cleavage of oversupplied acyl-CoA to produce free fatty
642 acids, considering the predicted role of the enzyme in fatty acid production in *E. coli*
643 (11). The excretion process of free fatty acids remains to be elucidated. Acyl-CoA is
644 thought to inhibit acetyl-CoA carboxylase (a complex of AccBC and AccD1), FasA, and
645 FasB, based on the knowledge in the related bacteria (52, 53). The repressor protein
646 FasR, combined with the effector acyl-CoA, represses the genes for these four proteins
647 (28). Repression and predicted inhibition are indicated by double lines. Arrows with
648 solid and dotted lines represent single and multiple enzymatic processes, respectively.
649 AccBC, acetyl-CoA carboxylase α-subunit; AccD1, acetyl-CoA carboxylase β-subunit;
650 FasA, fatty acid synthase IA; FasB, fatty acid synthase IB; Tes, acyl-CoA thioesterase;
651 FadE, acyl-CoA dehydrogenase; EchA, enoyl-CoA hydratase; FadB, hydroxyacyl-CoA
652 dehydrogenase; FadA, ketoacyl-CoA reductase; PM, plasma membrane; OL, outer
653 layer.

654

655 **FIG 2** Oleic acid-producing abilities of strains PAS-15, PC-33, and PCC-6. The three
656 strains, as well as wild-type ATCC 13032, were cultivated on MM agar pieces. After
657 cultivation for 2 days, the agar pieces were transferred onto bioassay plates containing
658 the oleic acid auxotroph OLA-15 as the indicator strain. The plates were incubated for 1

day at 30ºC. The images show one representative result from three independent experiments. Arrows represent the lineage relationship. Tween 40 and cerulenin were used as the potential specific inhibitors of fatty acid biosynthesis in *C. glutamicum* to induce oleic acid-producing mutants. Cerulenin^{L}, resistance to a relatively low concentration of cerulenin; Cerulenin^{H}, resistance to a relatively high concentration of cerulenin.

FIG 3 Three specific mutations identified in the oleic acid-producing mutants. Locations of mutations *fasR20*, *fasA63*^{up}, and *fasA2623* are indicated by dotted lines. The order in which these mutations arose is shown by circled digits. The *fasR20* mutation is located at nucleotide position 59 in the *fasR* gene (gray gene). The *fasA63*^{up} mutation is located at 63 bp upstream of the *fasA* gene. The nucleotide sequence of its surrounding region is also shown. The *fasA63*^{up} mutation is indicated by the larger character than those of its neighbors. The FasR-biding site *fasO* is boxed (28). The -10 and -35 regions of a potential promoter of *fasA* are underlined, and the transcriptional start site is also indicated by a bold and underlined character (28). Hatched boxes (boxes A-G) along the *fasA* gene represent nucleotide regions for putative catalytic domains involving in fatty acid synthesis (29, 48). A white part within box G represents a region for a motif sequence (PROSITE motif PS00606) for a 3-ketoacyl-ACP synthase active site. The *fasA2623* mutation is located within the motif. Box A represents a region for acetyl-CoA transferase, box B for enoyl-ACP reductase, box C for 3-ketoacyl-ACP dehydratase, box D for malonyl/palmitoyl transferase, box E for a substrate binding site of ACP, box F for 3-ketoacyl-ACP reductase, and box G for 3-ketoacyl-ACP synthase. The genes whose expression is thought to depend on FasR (28) are painted in black.

683

684 **FIG 4** Reconstitutions of defined mutations in the wild-type genome and their effects
685 on oleic acid production. Wild-type ATCC 13032 carrying the mutations *fasR20*,
686 *fasA63*^{up}, *fasA2623*, and Δ*fasR* separately or in combination were examined for their
687 abilities to produce oleic acid by using the same agar piece assay as conducted in Fig. 2.
688 The images show one representative result from three independent experiments. Plus
689 and minus signs represent the presence and absence of the corresponding mutation in
690 the wild-type background, respectively. The Δ*fasR* strain carries no other mutation,
691 except for the deleted *fasR* gene.

692

693 **FIG 5** Relative mRNA levels of the fatty acid biosynthesis genes in wild-type ATCC
694 13032 carrying the mutations *fasR20*, Δ*fasR*, and *fasA63*^{up} separately or in combination.
695 Total RNAs were prepared from cells grown to the early exponential phase (OD_{660} =
696 approximately 2.5) in MM medium. Aliquots of RNAs were reverse-transcribed, and
697 subjected to quantitative PCR. The transcript levels of *fasA* (white bars), *accD1* (black
698 bars), *accBC* (hatched bars), and *fasB* (dotted bars) were standardized to the constitutive
699 expression level of 16S rRNA. The transcript levels in wild-type ATCC 13032 were set
700 to 1.0. Data represent mean values from three independent cultures, and the standard
701 deviation from the mean is indicated as error bars.

702

703 **FIG 6** Time course of growth and glucose consumption in wild-type ATCC 13032 and
704 strain PCC-6. The two strains were cultivated in 30 ml of MM medium by rotary
705 shaking. Symbols: ●, growth of wild-type ATCC 13032; ■, growth of strain PCC-6; ○,
706 residual glucose in ATCC 13032; □, residual glucose in strain PCC-6. Values are means

of replicated cultures, which showed <5% differences between each other. Arrows indicate the time points at which culture supernatants were prepared for lipid analysis.

710 TABLE 1 Lipid production by wild-type ATCC 13032 and strain PCC-6[a]

Lipid	Wild		Strain PCC-6	
	Production (mg/liter)	Weight percentage (%)	Production (mg/liter)	Weight percentage (%)
Free fatty acid				
C15:1	1.61 ± 0.04	50.00 ± 0.16	2.93 ± 0.06	1.05 ± 0.02
C16:0	-	-	46.93 ± 2.03	16.76 ± 0.22
C16:1	0.71 ± 0.04	21.95 ± 0.68	6.39 ± 0.21	2.28 ± 0.00
C18:0	-	-	12.35 ± 0.46	4.41 ± 0.03
C18:1	0.90 ± 0.01	28.06 ± 0.84	208.10 ± 5.67	74.34 ± 0.23
C20:0	-	-	2.50 ± 0.06	0.89 ± 0.11
C20:1	-	-	0.77 ± 0.03	0.28 ± 0.00
Total	3.21 ± 0.06	100.00 ± 0.00	279.95 ± 8.50	100.00 ± 0.00
Phospholipid				
DPG	9.76 ± 0.47	100.00 ± 0.00	43.18 ± 1.84	100.00 ± 0.00
Total	9.76 ± 0.47	100.00 ± 0.00	43.18 ± 1.84	100.00 ± 0.00

711 [a]Culture supernatants were prepared at the points indicated by arrows in Fig. 6, and then
712 subjected to lipid analysis. The amounts of lipids were determined using two
713 independent cultures performed in Fig. 6. Values are represented as mean ± standard
714 deviation. DPG indicates diphosphatidylglycerol. Other phospholipids (e.g.,
715 phosphatidylinositol, phosphatidylglycerol, and phosphatidic acid) were not detected in
716 either strain. -, not detected.
717
718

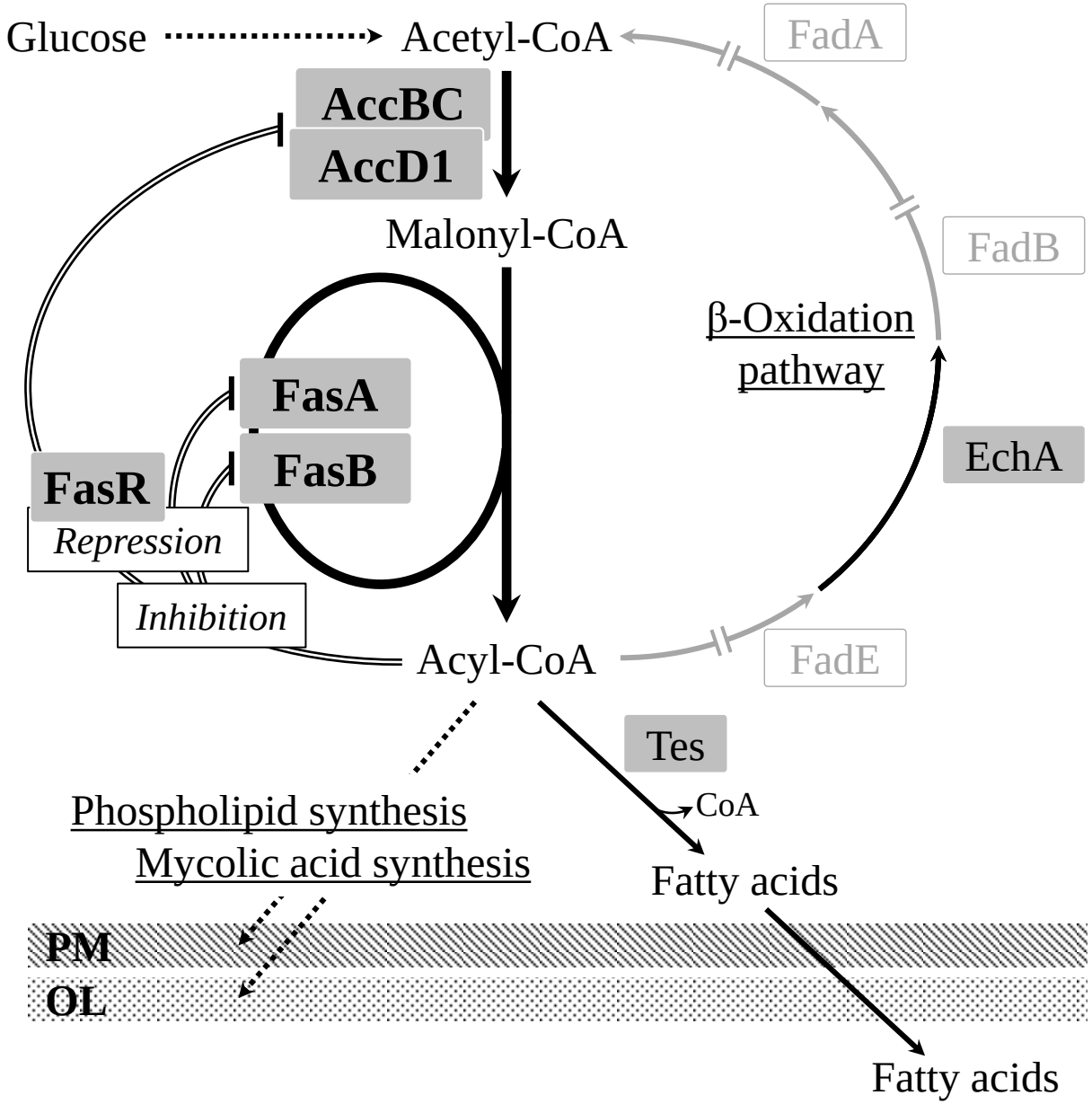
Glucose
Acetyl-CoA
AccBC
AccD1
Malonyl-CoA
FasA
FasB
FasR
Repression
Inhibition
Acyl-CoA
FadA
FadB
β-Oxidation pathway
EchA
FadE
Tes
CoA
Fatty acids
Phospholipid synthesis
Mycolic acid synthesis
PM
OL
Fatty acids

FIG. 1. Takeno

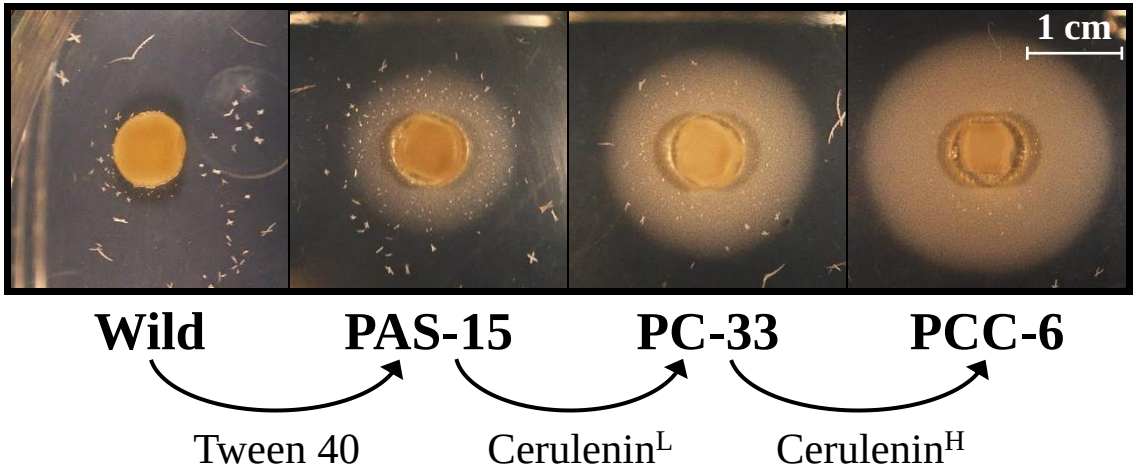

FIG. 2. Takeno

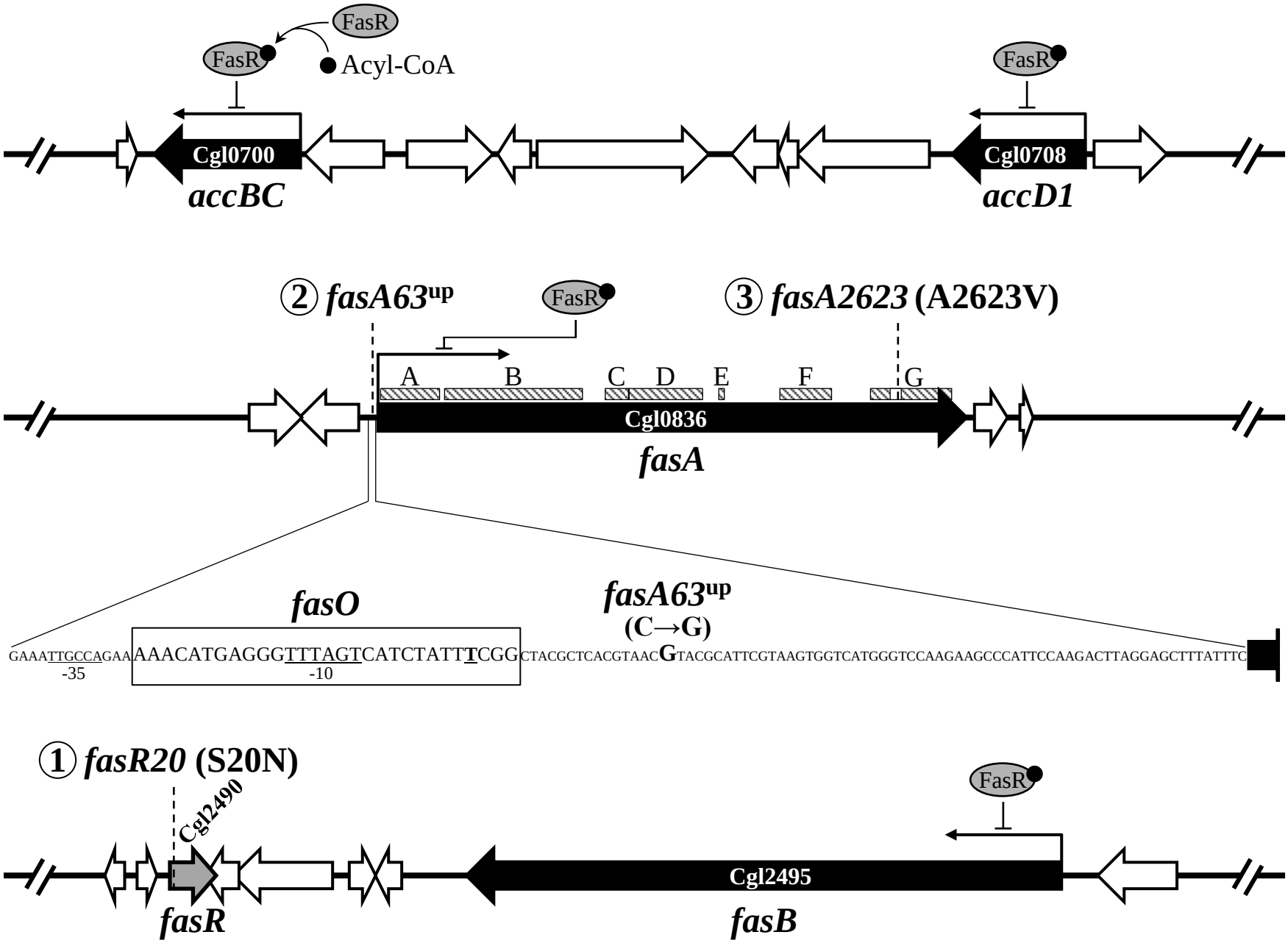
FasR
FasR
Acyl-CoA
FasR
FasR
Cgl0700
accBC
Cgl0708
accD1
② fasA63up
FasR
③ fasA2623 (A2623V)
A
B
C
D
E
F
G
Cgl0836
fasA
fasO
fasA63up
(C→G)
GAAATTGCCAGAAAAACATGAGGGTTTAGTCATCTATTTCGGCTACGCTCACGTAACGTACGCATTCGTAAGTGGTCATGGGTCCAAGAAGCCCATTCCAAGACTTAGGAGCTTTATTTC
-35
-10
① fasR20 (S20N)
Cgl2490
FasR
Cgl2495
fasR
fasB

FIG. 3. Takeno

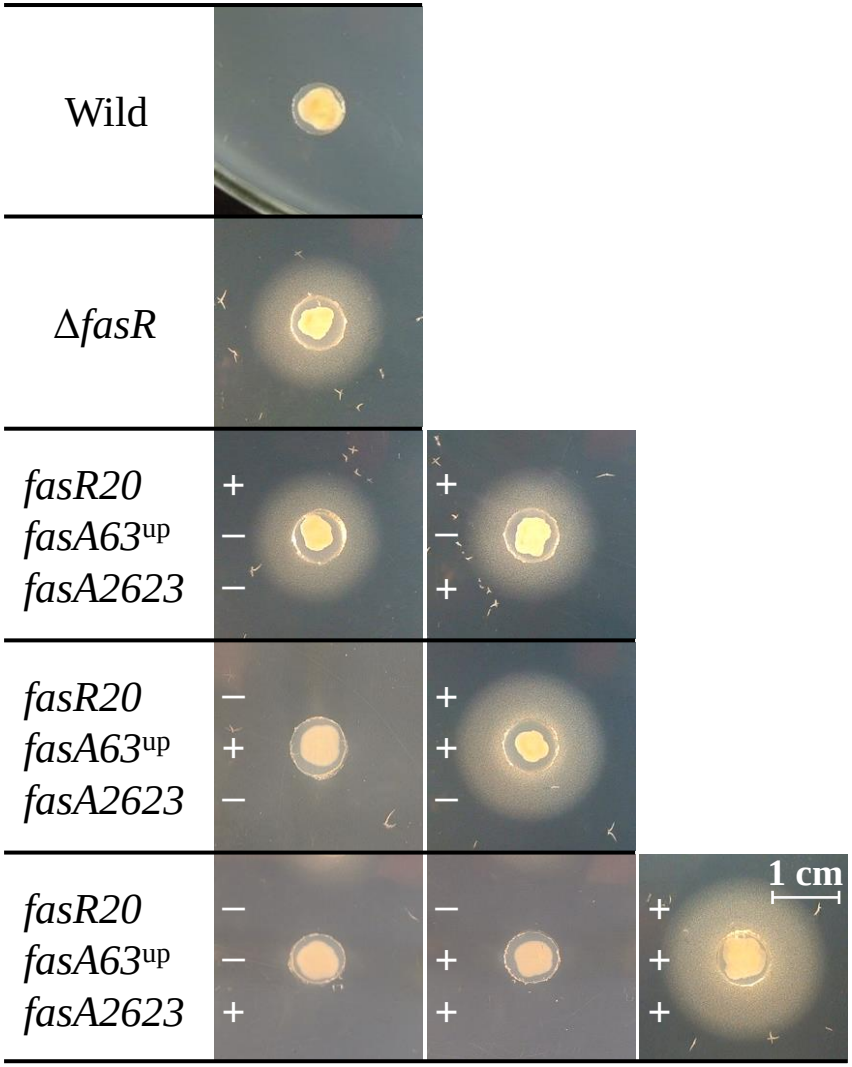
Wild
ΔfasR
fasR20
fasA63up
fasA2623
+
−
−
+
−
+
fasR20
fasA63up
fasA2623
−
+
−
+
+
−
fasR20
fasA63up
fasA2623
−
−
+
−
+
+
+
+
+
1 cm

FIG. 4. Takeno

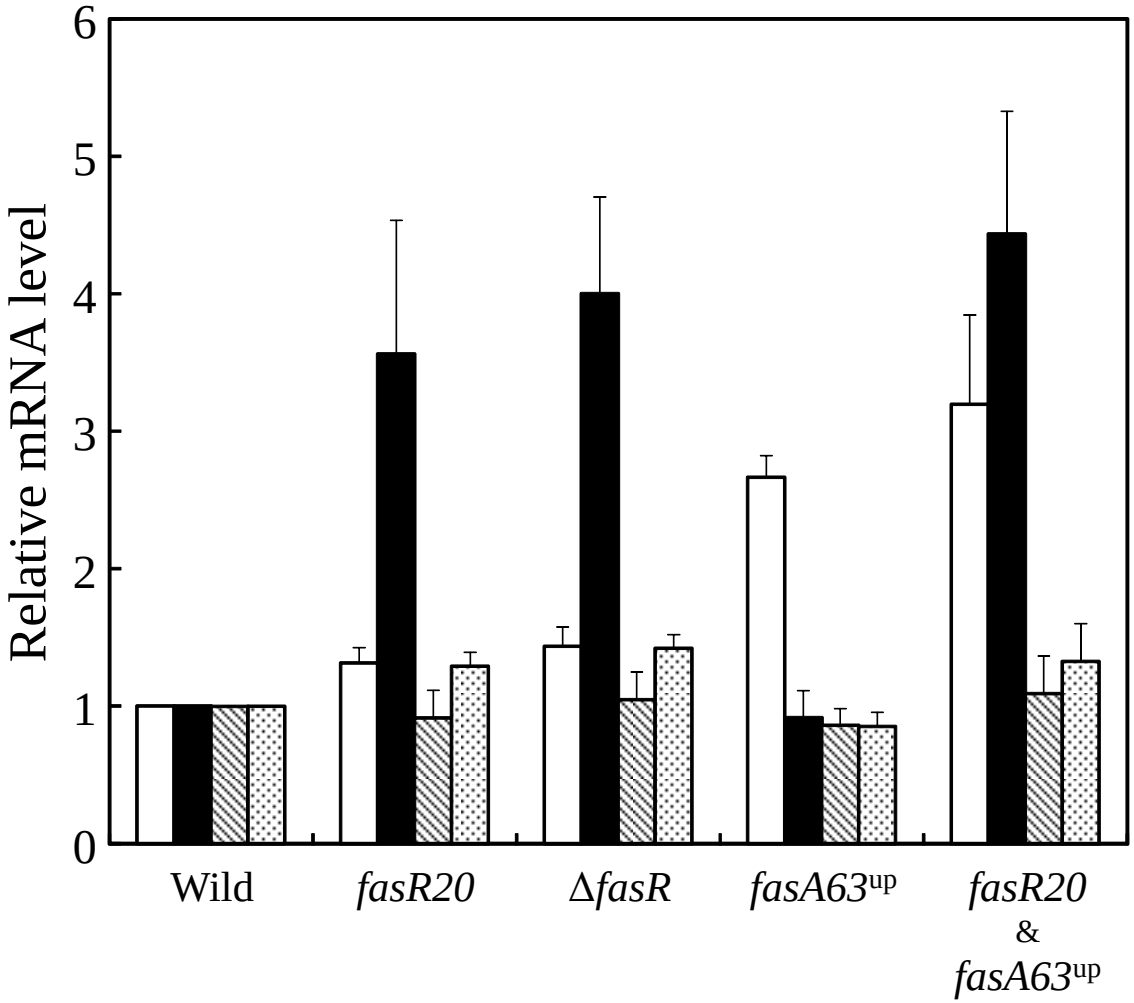
Relative mRNA level
6
5
4
3
2
1
0
Wild
fasR20
ΔfasR
fasA63up
fasR20
&
fasA63up

FIG. 5. Takeno

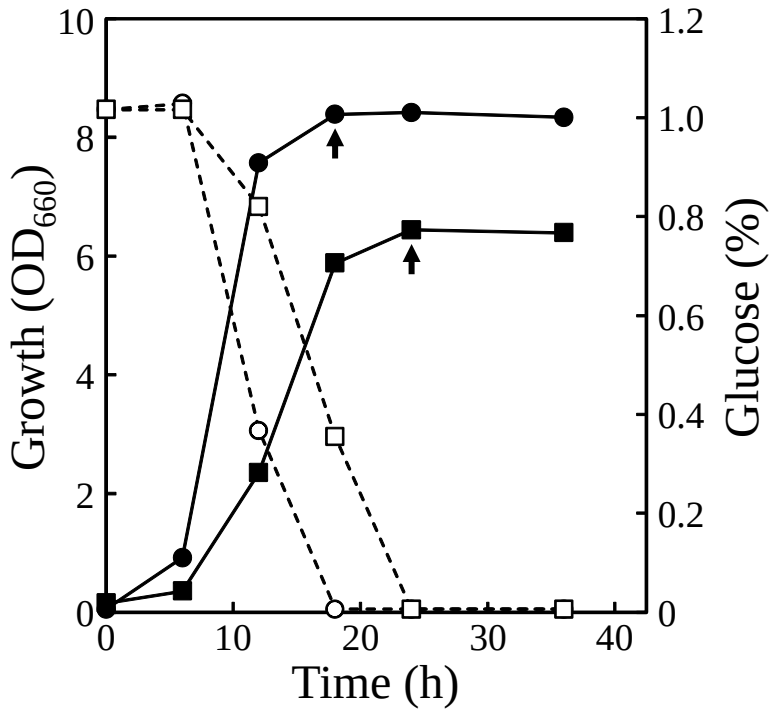
Growth (OD660)
Glucose (%)
Time (h)
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
1.0
1.2
0
10
20
30
40

FIG. 6. Takeno