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**Title**

# The *accD3* gene for mycolic acid biosynthesis as a target for improving fatty acid production by fatty acid-producing *Corynebacterium glutamicum* strains

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**Abstract**

We have recently developed *Corynebacterium glutamicum* strains that produce free fatty acids in culture supernatant due to enhanced fatty acid biosynthesis. Of these producing strains, the basic producer PAS-15 has a defect in the gene for a fatty acid biosynthesis repressor protein, and the advanced producer PCC-6 has two additional mutations to augment the production by strain PAS-15. The aim of the present study was to obtain novel genetic traits for improving fatty acid production by these producers. A new mutant with increased production derived from strain PAS-15 had a missense mutation in the *accD3* gene (mutation *accD3*$^{A433T}$), which is involved in the biosynthesis of mycolic acids that are cell envelope lipids of *C. glutamicum*, as the causal mutation. Mutation *accD3*$^{A433T}$ was verified to reduce the AccD3 enzymatic activity and increase fatty acid production in strain PAS-15 by 1.8-fold. Deletion of the *accD3* gene in strain PAS-15, which was motivated by the characteristic of mutation *accD3*$^{A433T}$, increased fatty acid production by 3.2-fold. Susceptibility of strain PAS-15 to vancomycin was significantly increased by *accD3* gene deletion and by mutation *accD3*$^{A433T}$ to the intermediate level, suggesting that the cell envelope permeability barrier by mycolic acids is weakened by this engineering. Furthermore, mutation *accD3*$^{A433T}$ also increased fatty acid production in strain PCC-6 by 1.3-fold. These increased production levels were suggested to be involved not only in the redirection of carbon flux from mycolic acid biosynthesis to fatty acid production but also in the permeability of the cell envelope.

**Keywords** *Corynebacterium glutamicum*· fatty acid production· *accD3*· mycolic acid· cell envelope permeability barrier

## Introduction

*Corynebacterium glutamicum* is an industrially important microorganism that is widely used for the production of various amino acids, including L-glutamate and L-lysine (Ikeda and Takeno 2013). Recently, the strain has been developed for the production of a variety of useful substances other than amino acids (Becker and Wittmann 2012; Heider et al. 2012; Plassmeier et al. 2016).

Fatty acid-producing strains of *C. glutamicum*, which has a unique style of fatty acid synthesis compared to common bacteria (Fig. 1), were constructed in our previous study with an objective to provide platforms for fatty acid-derived substance production (Takeno et al. 2013). There, we identified the specific mutations useful for fatty acid production by *C. glutamicum*: mutations *fasR20*, *fasA63*$^{up}$ and *fasA2623*. The mutation *fasR20*, which causes functional deficiency of a fatty acid biosynthesis repressor protein FasR, was verified to be of primary importance because the mutation confers fatty acid production on wild-type *C. glutamicum*. The mutation results in both increased carbon flow down the fatty acid biosynthetic pathway and excretion of the oversupplied fatty acid into the medium. This is reasonably explained by the fact that mutation *fasR20* increases the expression level of *accD1* for a β-subunit of acetyl-CoA carboxylase by 3.6-fold and of genes for two type I fatty acid synthases, *fasA* and *fasB*, by 1.3-fold. The *fasA63*$^{up}$ mutation, which is located upstream of the *fasA* gene, was demonstrated to increase the expression level of the *fasA* gene by 2.7-fold; and the *fasA2623* mutation, which is located in a 3-ketoacyl-ACP synthase active site of FasA, was supposed to alleviate the feedback inhibition. Mutations *fasA63*$^{up}$ and *fasA2623*

were shown to augment fatty acid production in the *fasR*-mutation background. These three mutations were obtained by analyzing a series of fatty acid-producing strains, PAS-15 and PCC-6, which were spontaneously isolated using two inhibitors of fatty acid biosynthesis (Takeno et al. 2013). Strain PAS-15, in which mutation *fasR20* was found, was derived as a mutant resistant to a palmitic acid surfactant Tween 40 from wild-type ATCC 13032. Strain PCC-6, in which mutations *fasA63*$^{up}$ and *fasA2623* were found in addition to *fasR20*, was isolated as a cerulenin-resistant mutant from strain PAS-15 via two rounds of selection using only cerulenin. Strain PCC-6, the most advanced producer thus far in our work, produces approximately 280 mg/L of fatty acids from 1% glucose within 24 h, but the production level of this strain remains less than 10% of the theoretical yield (Takeno et al. 2013).

The main aim of the present study is to obtain novel genetic trait(s) that can confer increased fatty acid production on the advanced producer PCC-6 so as to construct a forward-looking host for practical producers in the future. The approach previously employed to obtain useful genetic traits for fatty acid production, namely, the repeated use of limited kinds of fatty acid synthesis inhibitors in a situation where no other effective agents had been found during our screening, was no longer directly applicable to strain PCC-6 due to its already conferred high-level resistance. Therefore, the strategies employed in the present study were (i) to isolate cerulenin-resistant mutants with increased fatty acid production from the basic producer PAS-15 (the grandparental strain of PCC-6) to which cerulenin is still applicable, (ii) to identify the novel mutations causal to the increased production, and (iii) to verify whether the mutations increase fatty acid production by the advanced strain PCC-6. This approach will clearly indicate target genes for the increased production of fatty acids.

92 Characterization of these mutations will indicate what kind of manipulation should be
93 carried out on the gene to meet the purpose.
94 Here, we describe how a gene involved in the biosynthesis of mycolic acids,
95 the cell envelope lipids in *C. glutamicum*, can serve as the desired target for increasing
96 fatty acid production by these producers, and that the resulting increased production is
97 involved not only in a redirection of carbon flux from mycolic acid biosynthesis to fatty
98 acid production but also in permeability of the cell envelope in *C. glutamicum*.
99
100 **Materials and methods**
101
102 **Bacterial strains**
103
104 All strains used in this study are derivative mutants of the wild-type *C. glutamicum*
105 strain ATCC 13032. Fatty acid-producing strains PAS-15 and PCC-6 were previously
106 described (Takeno et al. 2013). Details regarding these strains are described below.
107 Strain PAS-15 had been isolated as a spontaneous mutant that is resistant to Tween 40
108 from wild-type ATCC 13032. A mutation in strain PAS-15 had been identified to be a
109 G-to-A exchange at nucleotide position 59 in the *fasR* gene (Cgl2490, NCgl2404),
110 which leads to the replacement of Ser-20 with Asn (mutation *fasR20*). Strain PCC-6, the
111 strain with increased production of fatty acids compared to strain PAS-15, had been
112 isolated as a spontaneous mutant resistant to cerulenin from strain PAS-15 via two
113 rounds of selection using only cerulenin (Takeno et al. 2013). In addition to mutation
114 *fasR20*, two mutations in strain PCC-6 had been identified to be a C-to-G exchange at
115 63 bp upstream of the *fasA* gene (mutation *fasA63*$^{up}$; Cgl0836, NCgl0802) and a C-to-T

116 exchange at nucleotide position 7868 in the *fasA* gene, which leads to the replacement
117 of Ala-2623 by Val (mutation *fasA2623*). *C. glutamicum* OLA-15 (Takeno et al. 2013),
118 which was used as an indicator strain for the agar piece assay, is an oleic
119 acid-auxotrophic mutant derived by a round of mutagenesis from wild-type ATCC
120 13032. *E. coli* DH5α was used as a host for DNA manipulation.
121
122 **Media and culture conditions**
123
124 Minimal medium (MM) (Takeno et al. 2007), complete medium BY (Takeno et al.
125 2007), and LFG1-ASL medium were used to cultivate *C. glutamicum* strains. For
126 plasmid carriers, BY and LFG1-ASL media were added with kanamycin at the
127 concentrations of 20 and 50 mg/L, respectively. MM contained 1% glucose as the sole
128 carbon source. LFG1-ASL medium, a modified LFG1 medium (Ohnishi et al. 2003) in
129 which only ammonium sulfate was limited, consisted (per liter) of 10 g of glucose, 10 g
130 of corn steep liquor, 4.0 g of $(NH_4)_2SO_4$, 2 g of urea, 0.5 g of $KH_2PO_4$, 0.5 g of
131 $MgSO_4 \cdot 7H_2O$, and 0.3 mg of biotin (pH 7.0). For fatty acid production in liquid culture,
132 a 3-mL sample of the seed culture grown in BY medium to the mid-exponential phase at
133 30ºC was inoculated into a 300-mL baffled Erlenmeyer flask containing 30 mL of MM
134 or LFG1-ASL media, followed by cultivation at 30ºC on a rotary shaker at 200 rpm.
135
136 **Agar piece assays for oleic acid production**
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138 Microbiological assay for oleic acid was performed by an agar piece method, as
139 described previously (Takeno et al. 2013). After cultivation of tested strains on the MM

agar piece for 3 days, the agar pieces were transferred onto bioassay plates containing the oleic acid auxotroph OLA-15 as an indicator strain. The plates were incubated for 1 day at 30ºC.

**Plasmids**

Plasmid pCS299P (Mitsuhashi et al. 2004), a *C. glutamicum-E. coli* shuttle vector, was used to clone the PCR product. Plasmid pESB30 (Mitsuhashi et al. 2004), which is nonreplicative in *C. glutamicum*, is a vector for gene replacement in *C. glutamicum*. Plasmid pCaccD3 was used to constitutively express the native *accD3* gene (Cgl2870, NCgl2772) under the promoter of the endogenous *gapA* gene. To construct pCaccD3, the coding region of *accD3* was amplified using the primers PgapAaccD3FusF and accD3down200RKpnI with wild-type *C. glutamicum* ATCC 13032 genomic DNA as a template. On the other hand, the genomic region from -1 to -558 bp upstream of the *gapA* gene, which comprises its promoter, was amplified using the primers PgapAKpBgF and PgapAaccD3FusR. These two fragments were fused by PCR with the primers PgapAKpBgF and accD3down200RKpnI. The resulting 2.3-kb fragment was digested with *Kpn*I and then ligated to *Kpn*I-digested pCS299P to yield pCaccD3. The primer sequences used in this study are presented in Supplementary Table S1. All primers were designed based on the genomic sequence of *C. glutamicum* (BA000036), which is publicly available at http://www.genome.jp/kegg/genes.html.

**Strain construction**

Plasmid pCaccD3$^{A433T}$, which was used for the introduction of mutation *accD3*$^{A433T}$ into the *C. glutamicum* genome, was constructed as described below. The mutated *accD3* region was amplified using the primers accD3inn480FBamHI and accD3down600RBamHI with genomic DNA from strain #43, and the resulting 1.7-kb fragment was digested with *Bam*HI and then ligated to *Bam*HI-digested pESB30 to yield pCaccD3$^{A433T}$. The amplified fragment was verified by DNA sequencing. Introduction of the specific mutation into the *C. glutamicum* genome was accomplished using plasmid pCaccD3$^{A433T}$ via two recombinant events, as described previously (Ohnishi et al. 2002). The presence of the mutation was confirmed by allele-specific PCR and DNA sequencing.

For chromosomal deletion of the *accD3* gene, plasmid pCΔaccD3, which contains the internally deleted *accD3* gene, was constructed as described below. The 5' region of the *accD3* gene was amplified using primers accD3up700FBamHI and accD3delFusR, with wild-type *C. glutamicum* ATCC 13032 genomic DNA. Similarly, the 3' region of the gene was amplified using primers accD3delFusF and accD3down600RBamHI. The 5' and 3' regions were fused by PCR using primers accD3up700FBamHI and accD3down600RBamHI. The resulting 1.5-kb fragment contained the deleted *accD3* gene, which was shortened by an in-frame deletion from 1551 to 81 bp. The fragment was digested with *Bam*HI and then ligated to *Bam*HI-digested pESB30 to yield pCΔaccD3. Defined chromosomal deletion of the *accD3* gene was accomplished via two recombination events with the plasmid (Ohnishi et al. 2002).

**Identification of a mutation in strain #43**

The mutation in strain #43 was identified via a comparative genome analysis with its parental strain PAS-15. Whole genome sequencing of strains #43 and PAS-15 was performed by Hokkaido System Science Co., Ltd. (Hokkaido, Japan) using an Illumina HiSeq (Illumina, SanDiego, CA).

**Quantitative determination of free fatty acids**

The culture supernatant was prepared by removing cells by centrifugation at 10,000×*g* for 20 min. Total lipids, including free fatty acids, were extracted from 2 mL of the supernatant by the Bligh-Dyer method (Bligh and Dyer 1959). The resulting chloroform extract was evaporated under reduced pressure; suspended in a solvent containing 0.5 mL of toluene, 0.2 mL of methanol, and 1 mL of trimethylsilyldiazomethane; and then incubated at room temperature for 3 h for methyl-esterification of the free fatty acids. After the reaction, the mixture was evaporated under reduced pressure, dissolved in 1.0 mL of chloroform containing 0.005% methyl nonadenanoic acid as an internal standard, and applied to a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and an Rxi-5ms column (Restek Corporation, Bellefonte, PA). The column temperature was kept at 50ºC for 1 min and then ramped to 270ºC at a rate of 8ºC per min, followed by holding at 270ºC for 5 min. The injector and detector temperatures were held at 250ºC and 330ºC, respectively. Fatty acids were identified and quantified by authentic fatty acid methyl ester standards.

**Determination of minimum inhibitory concentrations (MICs) for antibiotics**

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213 Minimum inhibitory concentrations (MICs) for vancomycin and erythromycin were
214 determined using E-test strips (BioMérieux, Marcy-l'Étoile, France). Exponential
215 cultures grown in LFG1-ASL medium containing 1% glucose were diluted in the
216 prewarmed same medium containing 0.6% agar to $OD_{660}$ of 0.03. Five milliliters of
217 each cell diluent were poured onto the 1.5% agar plate containing 17 mL of the same
218 medium in a 90-mm petri dish. After drying, E-test strips were applied. The plates were
219 incubated at 30ºC. The determination of the MICs was performed according to the
220 manufacturer's instructions.

221

222 **Analysis**

223

224 Bacterial growth was monitored by measuring the optical density at 660 nm ($OD_{660}$) of
225 the culture broth with a Miniphoto 518 R spectrophotometer (Taitec, Saitama, Japan).
226 Glucose concentration was determined using an F kit D-glucose (Roche Diagnostics,
227 Basel, Switzerland). Dry cell weight (DCW) per liter of *C. glutamicum* strains was
228 determined using the following formula that was established in advance: DCW (g) per
229 liter = $OD_{660} \times 0.5007 - 0.4202$.

230

231 **Results**

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233 **Isolation of mutants resistant to cerulenin from strain PAS-15**

234

We attempted to isolate spontaneous cerulenin-resistant mutants from the basic producer PAS-15. Appropriate dilutions ($10^5$-$10^6$ cells/mL) of the strain PAS-15 culture were spread on MM agar plates containing the MIC of cerulenin (approximately 7.5 mg/L). After cultivation for several days, all colonies appeared on all of the plates (236 colonies), which were obtained at a frequency of $10^{-4}$, were isolated and examined in triplicate for the production of oleic acid by agar piece assay. The assays revealed that a strain designated #43 shows the highest production (Fig. 2). This strain was further analyzed as a promising mutant that carries a novel mutation.

**Identification of a mutation in strain #43**

We conducted comparative genomic analysis between strains #43 and PAS-15. The analysis revealed only one specific mutation, a G-to-A exchange at nucleotide position 1297 in the *accD3* gene (Cgl2870, NCgl2772), which led to the replacement of Ala-433 by Thr (designated mutation *accD3*$^{A433T}$). Introduction of mutation *accD3*$^{A433T}$ into the strain PAS-15 genome, which yielded strain PASA-3, resulted in the equivalent halo size to that by strain #43 and a larger halo than strain PAS-15 in an agar piece assay for oleic acid (Fig. 2).

The *accD3* gene has been reported to encode a β-subunit of acyl-CoA carboxylase, which is involved in mycolic acid biosynthesis (Fig. 1) (Gande et al. 2004). Mycolic acids, long-chain α-alkyl, β-hydroxy fatty acids, are specific constituents of the outer part of the cell envelope in *Corynebacterineae*, including corynebacteria, mycobacteria, nocardia, and rhodococci (Puech et al. 2001; Daffé 2005), and play a key role as an outer permeability barrier; they also confer resistance to antibiotics in

*Mycobacterium tuberculosis* and *C. glutamicum* (Gebhardt et al. 2007; Brennan and Nikaido 1995). Mycolic acids in *C. glutamicum* range from $C_{32}$ to $C_{36}$ (Radmacher et al, 2005), and are synthesized by a condensation reaction via polyketide synthase Pks13 (encoded by Cgl2871, NCgl2773) using two fatty acid molecules produced via the fatty acid synthase reaction (Gande et al. 2004). It has been reported that AccD2 (encoded by Cgl0707, NCgl0677) and AccD3 are involved in the carboxylation of the fatty acids for mycolic acid synthesis, and that disruption of either the *accD2* or *accD3* genes results in the complete loss of mycolic acid without affecting phospholipid biosynthesis (Gande et al. 2004).

**Characterization of mutation *accD3*$^{A433T}$**

To characterize mutation *accD3*$^{A433T}$ and to investigate the effects of the mutation on growth and overall fatty acid production, strains PAS-15, PASA-3, and PASA-3/pCaccD3, a strain carrying a plasmid to constitutively express the native *accD3* gene, were cultivated in LFG1-ASL liquid medium containing 1% glucose (Fig. 3), and free fatty acid analysis of the culture supernatant was conducted (Fig. 4). The reason for using LFG1-ASL medium instead of the MM we have used for fatty acid analysis was that an in-frame *accD3* deletion mutant of strain PAS-15 as another reference strain (used in the latter section of the present study) did not grow at all in MM (data not shown). As shown in Fig. 3, strains PAS-15, PASA-3, and PASA-3/pCaccD3 exhibited equivalent growth rates. After glucose was consumed (at the points indicated by the arrows in Fig. 3), the cells were removed by centrifugation and the resulting culture supernatant was subjected to fatty acid analysis. All three strains produced palmitic,

oleic, and stearic acids, but varied in the total amount of fatty acids (Fig. 4). Strain PASA-3 produced 1.8-fold more fatty acids (209.86 ± 7.26 mg/L) than strain PAS-15 (116.81 ± 4.32 mg/L). On the other hand, expression of the native *accD3* gene reduced fatty acid production by strain PASA-3 to the level of strain PAS-15 (strain PASA-3/pCaccD3; 112.06 ± 4.55 mg/L). These results suggest that mutation *accD3*$^{A433T}$ being responsible for increased fatty acid production damages the function of the AccD3 enzyme. It should be noted that, albeit differences in the total amount of fatty acids, fatty acid composition (mol%) was almost unchanged among strains PAS-15 (Palmitic acid, 76.4%; Oleic acid, 12.0%; Stearic acid, 11.6%), PASA-3 (Palmitic acid, 75.6%; Oleic acid, 12.7%; Stearic acid, 11.7%), and PASA-3/pCaccD3 (Palmitic acid, 76.8%; Oleic acid, 11.5%; Stearic acid, 11.6%). These results suggest that the mutation does not influence the substrate specificity of the AccD3 enzyme, but reduces or abolishes the activity of this enzyme.

**Disruption of the *accD3* gene in strain PAS-15**

The *accD3* gene is likely to be a target for improving fatty acid production. Moreover, the above characteristics of mutation *accD3*$^{A433T}$ raised an expectation that a complete inactivation of the *accD3* gene increases fatty acid production more effective than mutation *accD3*$^{A433T}$. We constructed an in-frame *accD3*-deletion mutant of strain PAS-15 and examined its growth and fatty acid production under the same conditions as in Figs. 3 and 4. A markedly lower growth rate and a considerably lower final $OD_{660}$ were observed for strain PAS-15ΔaccD3 compared with those observed for strains PAS-15 and PASA-3 (Fig. 3). In terms of fatty acid production, strain PAS-15ΔaccD3

307 produced the same fatty acid species as strains PAS-15 and PASA-3 although it
308 produced 3.2- and 1.8-fold more total fatty acids (378.92 ± 16.14 mg/L) than strains
309 PAS-15 and PASA-3, respectively (Fig. 4). As in the case of strain PASA-3, the
310 expression of the native *accD3* gene reduced the production by strain PAS-15ΔaccD3 to
311 the level of strain PAS-15 (strain PAS-15/pCaccD3; 109.23 ± 4.52 mg/L). These results
312 demonstrate that a complete loss of the AccD3 enzymatic activity is considerably
313 effective in terms of final titer, aside from the damaged growth. Based on the order of
314 increasing production among strains PAS-15, PASA-3, and PAS-15ΔaccD, as well as
315 from comparison with their growth pattern, it was concluded that mutation *accD3*$^{A433T}$
316 does not abolish but reduces the enzymatic activity of AccD3.
317
318 **Susceptibility of strain PASA-3 to antibiotics**
319
320 Considering that mutation *accD3*$^{A433T}$ does not influence the substrate specificity of the
321 AccD3 enzyme, it is hard to assume that the mutation alters fatty acid composition of
322 mycolic acids. On the other hand, reduced activity of the AccD3 enzyme is likely to
323 decrease mycolic acid content. It has been reported that the absence of mycolic acids
324 results in a higher susceptibility to antibiotics (Gebhardt et al. 2007). Very recently, it
325 was reported in regard to *C. glutamicum* that disruption of the *sigD* gene encoding
326 sigma factor $\sigma^D$ leads to reduced expression of its regulon member involved in mycolic
327 acid biosynthesis, including the *accD3* gene, and results in a reduced amount of mycolic
328 acids, which accompanies enhanced susceptibility to antibiotics (Toyoda and Inui 2018).
329 Motivated by these previous studies, we examined strains PAS-15, PASA-3, and
330 PAS-15ΔaccD3 in terms of their MICs to the antibiotics vancomycin and erythromycin

using E-test strips (Fig. 5). Strain PAS-15ΔaccD3 had a lower MIC to vancomycin (0.094, 0.094, and 0.125 μg/mL; n = 3) than strain PAS-15 (0.190, 0.190, and 0.25 μg/mL; n = 3). In contrast, the MIC of strain PASA-3 to this antibiotic (0.125, 0.125, and 0.190 μg/mL; n = 3) was lower than that of strain PAS-15 and higher than that of strain PAS-15ΔaccD3. These results suggest that the mycolic acid content of strain PAS-15 was reduced by mutation *accD3*$^{A433T}$. This increased susceptibility to vancomycin observed for strain PASA-3 was also observed in the previous report on the *sigD* deletion strain of *C. glutamicum* (Toyoda and Inui 2018). There was no obviously determinable difference in the MIC to erythromycin between PAS-15 and PASA-3, which resembles the result that the *sigD* gene deletion leads to unchanged or slightly decreased susceptibility to this antibiotic (Toyoda and Inui 2018).

**Effect of mutation *accD3*$^{A433T}$ on growth and fatty acid production in strain PCC-6**

The deletion of the *accD3* gene is expected to increase fatty acid production by strain PCC-6. However, the deletion is also likely to severely damage the growth. The damaged growth at this stage is undoubtedly a drawback in the engineering toward practical producers in future, which, ideally, must have robust growth. In contrast, mutation *accD3*$^{A433T}$ increases fatty acid production, at least in strain PAS-15, without damaging the growth. Our primary interest in this study was to determine whether the mutation is also beneficial for increasing fatty acid production by the advanced producer PCC-6. To verify this, we constructed strain PCCA-3 by introducing mutation *accD3*$^{A433T}$ into the strain PCC-6 genome, and examined the resulting strain for its growth and fatty acid production using LFG1-ASL and MM media, both containing 1%

355 glucose. In LFG1-ASL medium, as in the case of strain PAS-15 and PASA-3, strain
356 PCCA-3 exhibited a similar growth curve to that of the parental strain PCC-6 (Fig. 6).
357 For fatty acid production, strain PCCA-3 produced 1.3-fold more total fatty acids
358 (238.22 ± 8.55 mg/L) than strain PCC-6 (180.47 ± 7.61 mg/L) in LFG1-ASL medium
359 (Fig. 7). On the other hand, in MM, a slightly lower final $OD_{660}$ was observed for strain
360 PCCA-3 compared with that of strain PCC-6, but glucose was consumed by 24 h in the
361 both strains (Fig. 6). Strain PCCA-3 produced 1.2-fold more total fatty acids (322.23 ±
362 15.09 mg/L) than strain PCC-6 (260.23 ± 11.27 mg/L) in MM (Fig. 7). These results
363 demonstrate that mutation *accD3*$^{A433T}$ is applicable to the further engineering of the
364 advanced producer toward the more efficient production of fatty acids.
365
366 **Discussion**
367
368 In this report, we identified mutation *accD3*$^{A433T}$ responsible for increasing fatty acid
369 production by the basic producer PAS-15. The mutation also increased the fatty acid
370 production by the advanced producer PCC-6. These results demonstrate the validity of
371 the strategy in this study: improving the advanced producer, which is no longer easily
372 directly engineered by the method depending on the fatty acid synthesis inhibitors,
373 using the novel positive mutation found in the mutant newly obtained from its ancestor
374 to which the inhibitor is still applicable.
375 The total amount of fatty acids produced by strain PAS-15 was increased by
376 3.2-fold by the disruption of the *accD3* gene and by only 1.8-fold by mutation
377 *accD3*$^{A433T}$ (Fig. 4). Whereas the disruption had a great positive impact on fatty acid
378 production beyond the mutation, it prolonged the cultivation period required to

complete the production (Fig. 3). In contrast, mutation *accD3*$^{A433T}$ did not accompany growth retardation (Fig. 3). Taking the cultivation period into account (16 h for strains PAS-15 and PASA-3, and 36 h for strain PAS-15ΔaccD3), the fatty acid production rate (productivity) of the overall process resulted in the following order: 7.30 mg/L/h for strain PAS-15, 10.52 mg/L/h for strain PAS-15ΔaccD3, and 13.12 mg/L/h for strain PASA-3. Therefore, mutation *accD3*$^{A433T}$ seems more advantageous in terms of productivity than complete inactivation of the *accD3* gene. The positive effect of mutation *accD3*$^{A433T}$ is probably promising in advanced producers. In fact, strain PCCA-3 had 1.3- (14.89 mg/L/h) and 1.2-fold (13.42 mg/L/h) higher productivity in fatty acids than the parental strain, in LFG1-ASL and MM media, respectively (Figs. 6 and 7).

The decreased MIC of strain PASA-3 to vancomycin was judged to reflect the lower mycolic acid content caused by reduced activity of the AccD3 enzyme. We initially presumed that the reduced activity merely redirects fatty acids from mycolic acids into fatty acid production. However, we should now discuss the increased production from the following viewpoint: the facilitation of fatty acid excretion that results from weakening the mycolic acid barrier. The mycolic acid content of *C. glutamicum* has been reported to be 1.79 mmol/100 g of DCW (Bansal-Mutalik and Nikaido 2011). The main mycolic acids in *C. glutamicum* are $C_{32:0}$ and $C_{36:2}$ (Lanéelle et al. 2013), which have an average molecular weight of approximately 508 mg; therefore, 100 g of dry cells is estimated to contain 909 mg of mycolic acids. According to this, since the DCW of the strain PASA-3 cells per liter of LFG1-ASL culture is determined based on the value of $OD_{660}$ in Fig. 3 to be approximately 5.4 g/L, mycolic acids existing in the whole culture are estimated to be approximately 49 mg/L. The value,

403 however, can never explain the increase of as much as 93 mg/L of fatty acids that was
404 obtained by comparing the final titers in strains PAS-15 (116.81 ± 4.32 mg/L) and
405 PASA-3 (209.86 ± 7.26 mg/L). This gap also occurs between strains PCC-6 and
406 PCCA-3 regardless of the culture media, and is more evident for strain PAS-15ΔaccD3,
407 which is incapable of producing mycolic acids: the DCW of PAS-15ΔaccD3 cells per
408 liter of LFG1-ASL culture is estimated to be approximately 3.4 g/L, and an increase of
409 262 mg/L of fatty acids was observed between strains PAS-15 and PAS-15ΔaccD3 (Figs.
410 3 and 4). Mycolic acid in *C. glutamicum* is also known to be the permeability barrier
411 against substance excretion. In addition to the increased susceptibility to antibiotics, the
412 absence of mycolic acids leads to the increased production of both L-glutamate and
413 L-lysine by *C. glutamicum* due to the improved permeability of the cell wall (Gebhardt
414 et al. 2007). Accordingly, also in the present situation, the reduced content of mycolic
415 acids caused by mutation *accD3*$^{A433T}$ may improve excretion efficiency for fatty acids
416 and thereby contribute to fatty acid production. This idea is supported by the
417 observation that the amount of free fatty acids released extracellularly is increased by
418 the enhancement of cell membrane permeability in the producer of *Aspergillus oryzae*
419 (Tamano et al. 2017).
420 In conclusion, the *accD3* gene serves as the target for the engineering of fatty
421 acid producers of *C. glutamicum*, and not abolished but reduced activity of the AccD3
422 enzyme can reconcile growth and productivity. Moreover, whereas the permeability
423 barrier by mycolic acids in *C. glutamicum* has mainly been dealt with in production of
424 hydrophilic substances, such as L-lysine and L-glutamic acid, the present study indicates
425 that the barrier also functions against the production of hydrophobic long-chain fatty
426 acids. Judging from the fact that the effect of mutation *accD3*$^{A433T}$ to increase fatty acid

production remained at approximately 60% of that of the *accD3* gene deletion, there still seems room for improvement of *accD3*/AccD3. This needs to be addressed in future work. Besides low-level expression of the *accD3* gene using inducible expression system or by diminishing activity of its native promoter, construction of mutated AccD3 enzymes that have different amino acid residues at their amino acid position 433 may be effective. Any solution for the issue requires to be optimized so that growth and fatty acid production are well balanced.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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567
568 **Figure legends**
569

570 **Fig. 1** Lipid metabolism and its proposed regulatory mechanism in *C. glutamicum*.
571 Unlike the majority of bacteria, including *E. coli* and *B. subtilis*, coryneform bacteria,
572 such as members of the genera *Corynebacterium* and *Mycobacterium*, are known to
573 possess type I fatty acid synthase, a multienzyme that performs successive cycles of
574 fatty acid synthesis. In *C. glutamicum*, fatty acids are believed to be synthesized as
575 acyl-CoA (Kawaguchi and Okuda 1977) by two type I fatty acid synthases, FasA and
576 FasB, and the CoA derivatives are used for the synthesis of membrane phospholipids
577 and the outer layer component mycolic acids. The FasA enzyme produces mainly oleic
578 acid and palmitic acid with a relatively small amount of stearic acid whereas FasB
579 mostly synthesizes palmitic acid (Radmacher et el. 2005). Recently, the FasB enzyme
580 was reported to be involved in biosynthesis of the $C_8$ compound lipoic acid in this
581 organism (Ikeda et al. 2017). The Tes enzyme is assumed to be involved in the cleavage
582 of acyl-CoA to produce free fatty acids, considering the predicted role of the enzyme in
583 fatty acid production in *E. coli* (Cho and Cronan 1995). The process of free fatty acid
584 excretion remains to be elucidated. Mycolic acids are suggested to be synthesized by
585 condensation of α-carboxyl-acyl-CoA and acyl-AMP, which is catalyzed by polyketide
586 synthase Pks13 (Portevin et al. 2004), and the subsequent reduction of the resulting
587 keto-ester function to a hydroxyl group by CmrA (Lea-Smith et al. 2007). Whereas
588 acyl-AMP is assumed to be produced by acyl-CoA synthetase FadD32 from free fatty
589 acid (Portevin et al. 2005), α-carboxyl-acyl-CoAs are believed to be produced by
590 carboxylation of acyl-CoAs, which is catalyzed by an enzyme complex consisting of
591 two acyl-CoA carboxylase β-subunits, AccD2 and AccD3; a biotinylated α-subunit
592 AccBC (represented as BC); and an ε-subunit AccE (represented as E) (Gande et al.
593 2004; Gande et al. 2007). The AccBC and AccE are shared by the acetyl-CoA

carboxylase complex, including the β-subunit AccD1 (Gande et al. 2007). Three genes responsible for the β-oxidation of fatty acids are missing from the *C. glutamicum* genome (gray arrows) (Barzantny et al. 2012). Acyl-CoA is thought to inhibit acetyl-CoA carboxylase, FasA, and FasB based on knowledge of related bacteria (Erfle 1973; Morishima and Ikai 1987). The repressor protein FasR, combined with the effector acyl-CoA, represses the genes for *accD1*, *fasA*, and *fasB* (Nickel et al. 2010; Irzik et al. 2014). Repression and predicted inhibition are indicated by double lines. Arrows with solid and dotted lines represent single and multiple enzymatic processes, respectively. Tes, acyl-CoA thioesterase; CmrA, short-chain dehydrogenase Cgl2472; FadE, acyl-CoA dehydrogenase; EchA, enoyl-CoA hydratase; FadB, hydroxyacyl-CoA dehydrogenase; FadA, ketoacyl-CoA reductase; MA, mycolic acid; PM, plasma membrane; OL, outer layer.

**Fig. 2** Oleic acid production of strains PAS-15, #43, and PASA-3. These three strains and wild-type strain ATCC 13032 were cultivated on MM agar pieces. After cultivation for 3 days, the agar pieces were transferred onto bioassay plates containing the oleic acid auxotroph OLA-15 as an indicator. The plates were incubated for 1 day at 30ºC. The images show one representative result from three independent experiments.

**Fig. 3** Growth of strains PAS-15 (open circle), PASA-3 (open square), PAS-15ΔaccD3 (open triangle), PASA-3/pCaccD3 (solid square), and PAS-15ΔaccD3/pCaccD3 (solid triangle), and wild-type strain ATCC 13032 (solid circle). All strains were cultivated in 30 mL of LFG1-ASL medium containing 1% glucose in a 300-mL baffled Erlenmeyer flask at 30ºC with rotary shaking at 200 rpm. Values are means of three independent

618 cultures, which showed <5% difference from each other. Arrows indicate the time points
619 at which all of the glucose was consumed.
620
621 **Fig. 4** Fatty acid production by strains PAS-15, PASA-3, PAS-15ΔaccD3,
622 PASA-3/pCaccD3, and PAS-15ΔaccD3/pCaccD3. Culture supernatants were prepared at
623 the points indicated by the arrows in Fig. 3, and subjected to free fatty acid analysis.
624 The amounts of fatty acids were determined using three independent cultures performed
625 as described in the legend to Fig. 3. The other fatty acids not presented here were not
626 detected or only detected in trace amounts below 0.5 mg/L. Data represent mean value,
627 and the standard deviation from the mean is indicated as error bars.
628
629 **Fig. 5** Susceptibilities of strains PAS-15, PASA-3, and PAS-15ΔaccD3 to antibiotics.
630 Exponential cultures of these strains grown in LFG1-ASL medium containing 1%
631 glucose were diluted in the prewarmed same medium containing 0.6% agar to $OD_{660}$ of
632 0.03. Five milliliters of each cell diluent were poured onto the 1.5% agar plate
633 containing 17 mL of the same medium in a 90-mm petri dish. After drying, E-test strips
634 of vancomycin (VA) and erythromycin (EM) were applied onto the surface of the plates.
635 The plates for strains PAS-15 and PASA-3 were incubated at 30ºC for 48 h and for
636 strain PAS-15ΔaccD3 for 96 h.
637
638 **Fig. 6** Growth of strains PCC-6 and PCCA-3. Strains PCC-6 (circle) and PCCA-3
639 (square) were cultivated in 30 mL of LFG1-ASL media (solid symbols) and MM (open
640 symbols), both containing 1% glucose, in 300-mL baffled Erlenmeyer flasks at 30ºC
641 with rotary shaking at 200 rpm. Values are means of three independent cultures, which

642 showed <5% difference from each other. Arrows indicate the time points at which all of
643 the glucose was consumed.
644
645 **Fig. 7** Fatty acid production by strains PCC-6 and PCCA-3. Culture supernatants were
646 prepared at the points indicated by the arrows in Fig. 6, and subjected to fatty acid
647 analysis. The amounts of fatty acids were determined using three independent cultures
648 performed as described in the legend to Fig. 6. The other fatty acids not presented here
649 were not detected or only detected in trace amounts below 0.5 mg/L. Data represent
650 mean value, and the standard deviation from the mean is indicated as error bars.

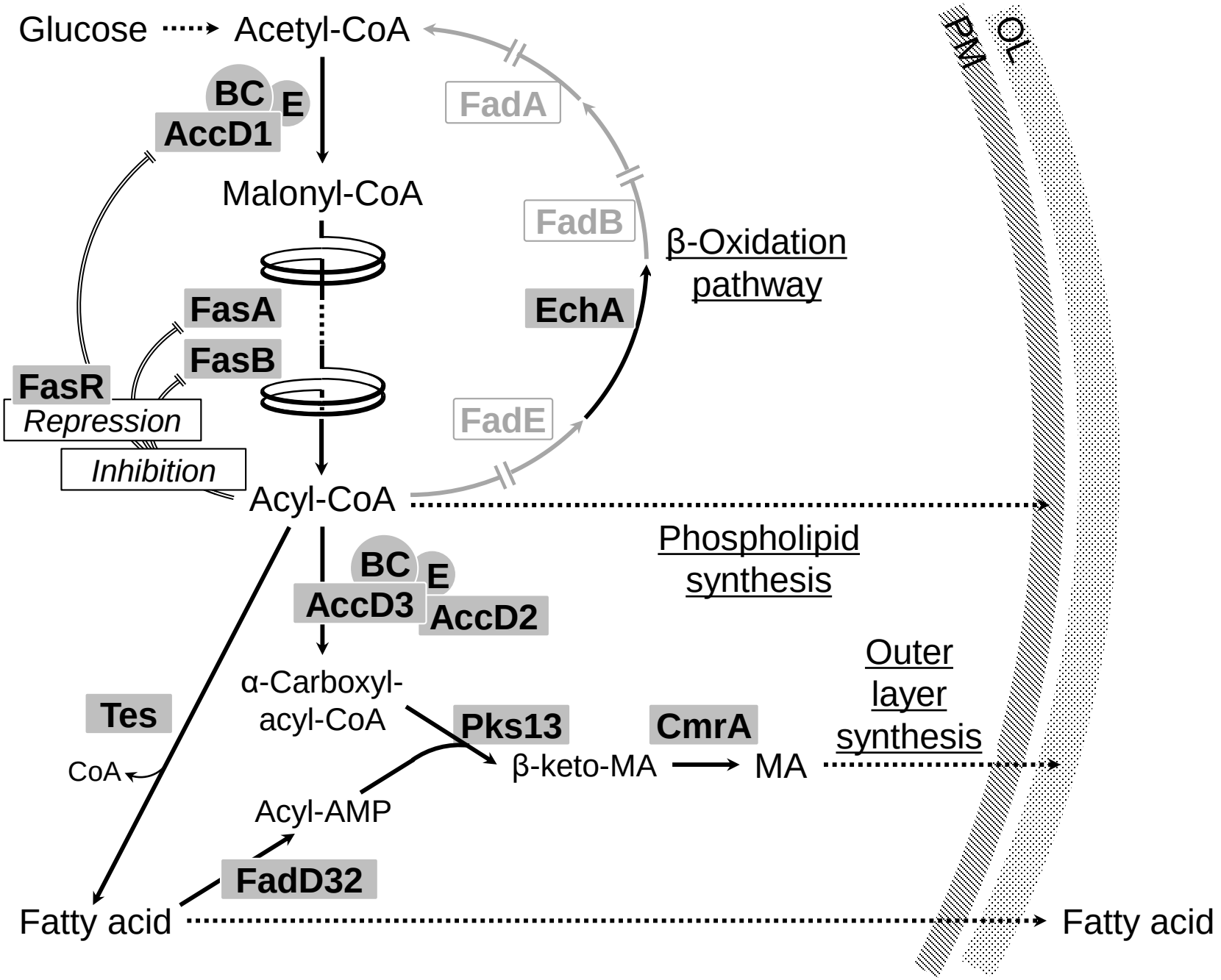
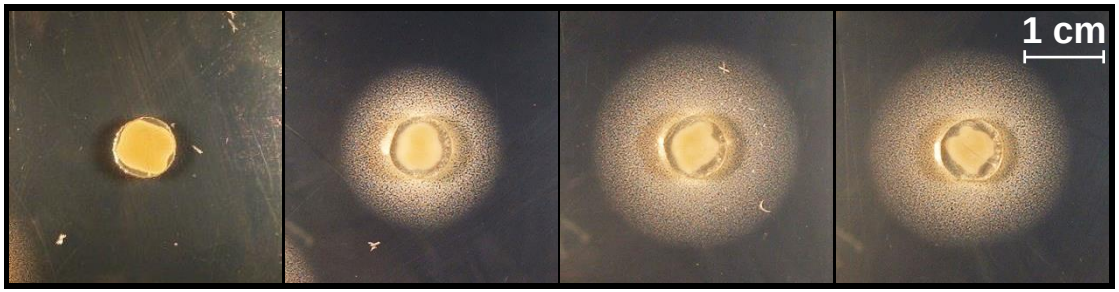



Fig. 1 Takeno et al.

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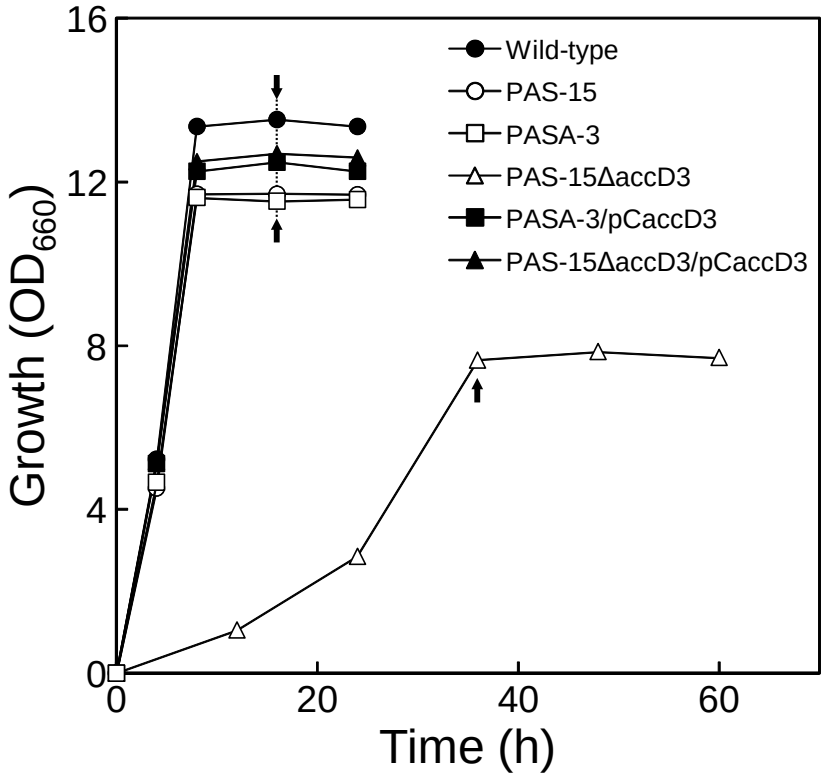


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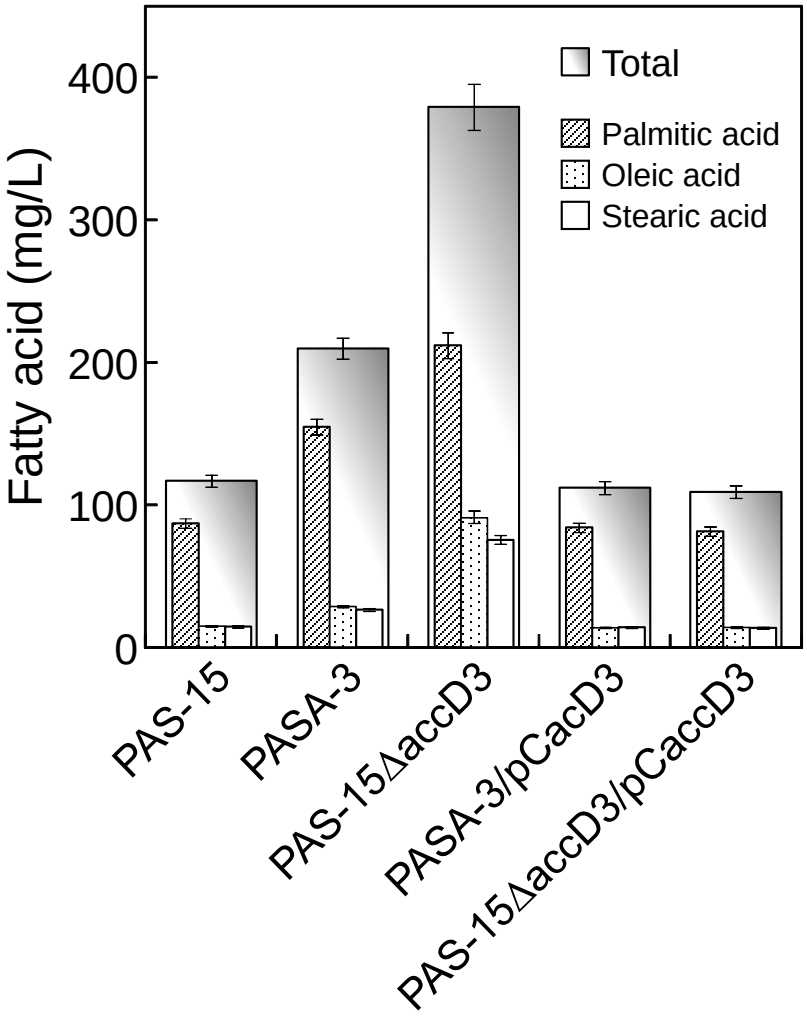


Fig. 4 Takeno et al.

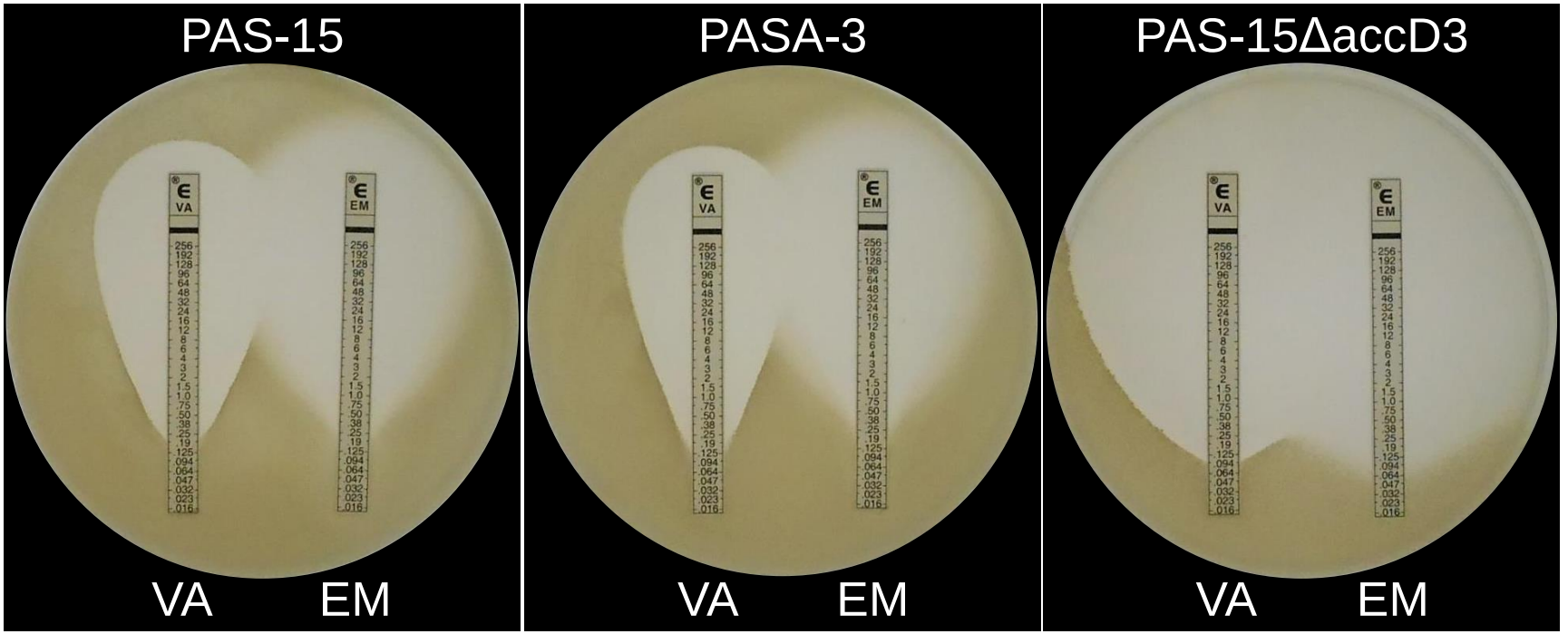


Fig. 5 Takeno et al.

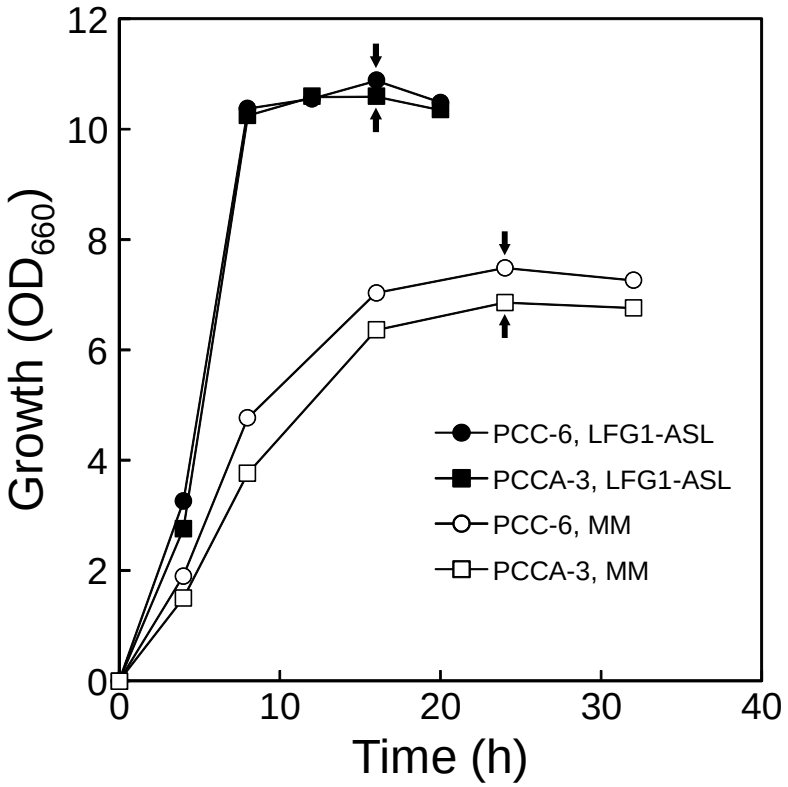


Fig. 6 Takeno et al.

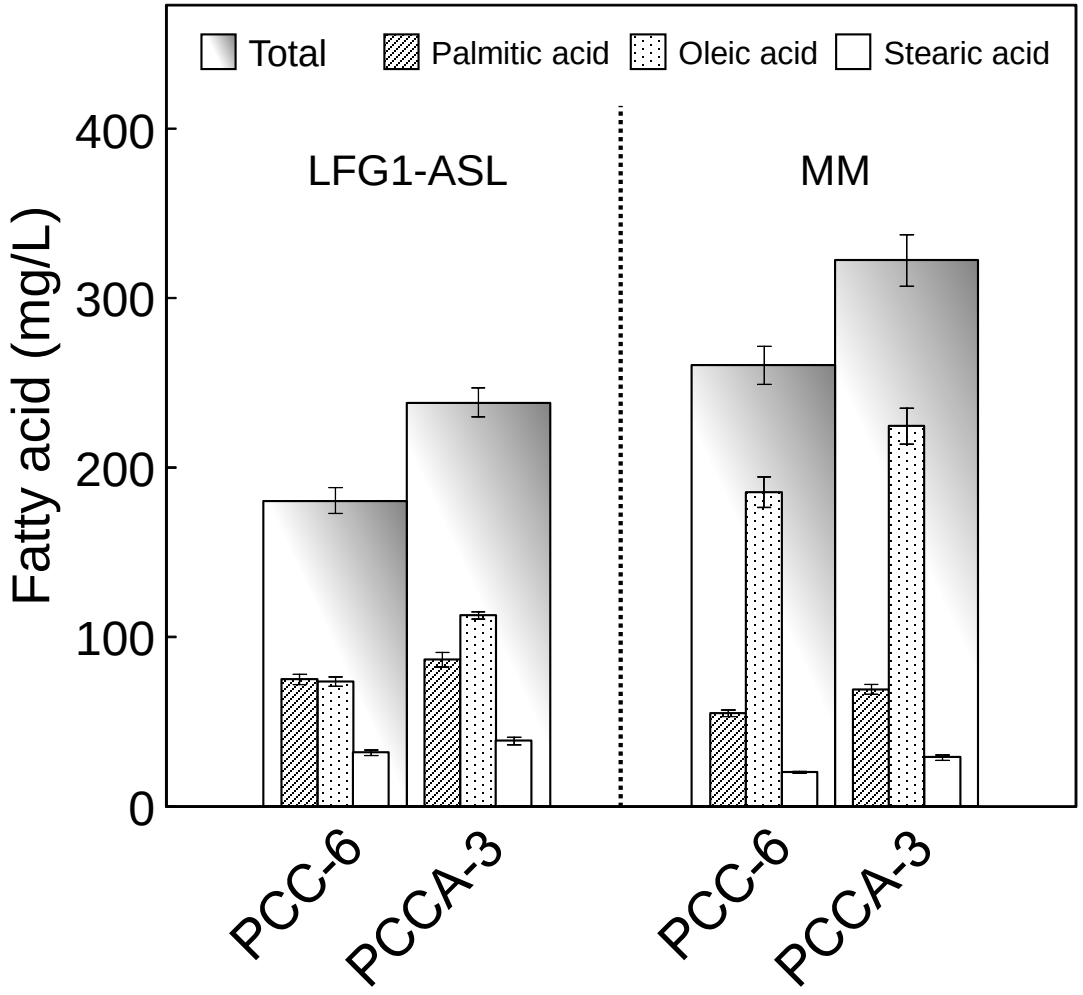


Fig. 7 Takeno et al.