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Corynebacterium glutamicum CgynfM encodes a dicarboxylate transporter applicable to succinate production

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After deleting the gene encoding succinate dehydrogenase, *Corynebacterium glutamicum* can produce succinate and a considerable amount of acetate and pyruvate as by-products from glucose metabolism, under aerobic conditions. Recently, we identified *ynfM* in *Pantoea ananatis* (*PaynfM*) as a gene encoding a dicarboxylate transporter and found a homologous gene (*CgynfM*) in *C. glutamicum*. In this study, we examined dicarboxylate production using *C. glutamicum* strains expressing *CgynfM*. When *C. glutamicum* expressing the *CgynfM* gene was cultured under aerobic conditions, the sugar-consumption rate increased significantly, succinate accumulation increased from 66 mM to 110 mM, and pyruvate and acetate co-production decreased significantly. Pyruvate decreased from 120 mM to 6.2 mM, and acetate decreased to undetectable level. CgYnfM restored succinate production under anaerobic conditions in *C. glutamicum* strain AJ110655Δ*sucE1*, in which the gene encoding the major succinate exporter (*sucE1*) was deleted. *CgynfM* expression also increased α-ketoglutarate production from 5.1 mM to 24 mM under anaerobic conditions. Collectively, these results suggest that YnfM from *C. glutamicum* functions as a dicarboxylate transporter that is applicable to the succinate production.

[**Key words:** *Corynebacterium glutamicum*; Dicarboxylate transporter; Succinate production; α-Ketoglutarate production; Anaerobic conditions; Aerobic conditions]

Organic acids have long been used as raw materials in the food, chemical, and pharmaceutical industries. Recently, ethyl lactate and diethyl succinate, which are lactic acid and succinic acid (succinate) esters, respectively, have gained increasing attention because they are environmentally benign solvents (1). Moreover, there is a growing market for lactate and succinate polymers as alternative raw materials for producing biodegradable plastics (2,3).

Succinate is produced via the action of anaplerotic enzymes and the reductive arm of the tricarboxylic acid (TCA) cycle pathway by anaerobic microorganisms such as *Anaerobiospirillum succiniciproducens* (4), *Actinobacillus succinogenes* (5), and *Mannheimia succiniciproducens* (6). In the absence of oxygen and exogenous electron acceptors, the facultative anaerobes *Escherichia coli* and *Corynebacterium glutamicum* can produce succinate from sugars via the reductive TCA cycle (7–10). Even though anaerobic processes for succinate production have resulted in high yields and titers, some problems remain to be solved. For example, the optimal redox balance was previously found to be limited (11,12). Aerobic cell culture conditions were reported to have advantages over anaerobic conditions, such as higher biomass generation, and faster carbon throughput and product formation (12,13). Thus, several aerobic platforms have been designed and constructed. Several aerobic succinate-producing *E. coli* strains were designed that could achieve high succinate yields, using various sugars (11,13). The yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (which showed high tolerance toward acidity) were also genetically modified for succinate production, but had low productivities (14,15). Recently, it was reported that a *C. glutamicum* strain lacking succinate dehydrogenase (SDH) showed greater potential for aerobic succinate production than other producers with equivalent genetic backgrounds (16). *C. glutamicum* lacking the SDH also produced high amounts of acetate as a by-product (15). Although acetate production could be largely reduced by deleting genes for all known acetate-production pathways (*pta-acks*, *pqo*, and *cat*) and expressing *acsA*, *gltA*, *ppc*, and *pyc*, a considerable amount of pyruvic acid was still produced (16).

We previously demonstrated that succinate transporters markedly impact fermentative production. First, we found that the *sucE1* gene, which belongs to the aspartate: alanine exchanger family (Transporter Classification [TC] system (17) 2.A.81), encodes a succinate exporter crucial for succinate production in *C. glutamicum* under anaerobic conditions (18,19). Next, we found that YjjP and YjjB, which belong to the ThrE family (TC 2.A.79), formed a succinate transporter (YjjPB) in *E. coli* and that the products of both genes were required for succinate export (20). Expression of the *yjjPB* genes enhanced succinate production in *Pantoea ananatis*

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under aerobic conditions, and their deletion decreased succinate production in *E. coli* by 70% under anaerobic conditions. Third, we identified the *yjjPB* homolog in *Enterobacter aerogenes* (*EayjjPB*) and showed that EaYjjPB constituted a dicarboxylate transporter (21). EaYjjPB expression enhanced succinate production two-fold in *E. aerogenes* under aerobic conditions. Under anaerobic conditions, EaYjjPB expression increased the succinate yield (g succinate·g glucose^{-1}) from 53% to 60% and its deletion decreased the succinate yield to 48%.

P. ananatis AJ13355 is a newly identified member of the Enterobacteriaceae family with promising biotechnological applications. This bacterium can grow at an acidic pH and is resistant to saturating concentrations of L-glutamic acid, making this organism a suitable host for L-glutamate production (22). Recently, we found that *ynfM* from *P. ananatis* (*PaynfM*) encodes a dicarboxylate transporter that is important for dicarboxylate production. When a *P. ananatis* succinate producer expressing the *PaynfM* gene was cultured under aerobic conditions, succinate production did not increase, but α-ketoglutarate production increased significantly from 6.8 mM to 21 mM. When a *P. ananatis* malate producer expressing the *PaynfM* gene was cultured under aerobic conditions, malate production increased from 3.7 mM to 16 mM (23). *C. glutamicum* has a gene encoding a protein (CgYnfM) homologous to PaYnfM. CgYnfM and PaYnfM share relatively high similarity in their amino acid sequences (identity, 39%; positives, 59%; gaps, 0.8%). This fact motivated us to characterize CgYnfM as a candidate protein involved in succinate transport in *C. glutamicum* and to evaluate its impact on succinate production by the bacterium. To this end, we demonstrated the expression of *CgynfM* gene in an engineered *C. glutamicum* led to the significant improvement of the titer and yield of succinate production under an aerobic condition although no obvious change was found in the succinate production in *P. ananatis* with *PaynfM* expression.

MATERIALS AND METHODS

Bacterial strains and culturing Details regarding the strains and plasmids used in this study are summarized in Table 1. Plasmids were introduced into *E. coli* and *C. glutamicum* by electro-transformation (24). *E. coli* cells were grown in Luria–Bertani (LB) broth (24) at 37°C. *C. glutamicum* cells were grown in CM-Dex medium (25) at 31.5°C. Kanamycin was used to select for transformants and maintain plasmids at 25 mg L^{-1} in *C. glutamicum*, and 40 mg L^{-1} in *E. coli*. All bacteria were shaken in test tubes or Sakaguchi flasks capped with silicosen (Shin-Etsu Polymer, Tokyo, Japan) during growth, except for experiments involving succinate production.

Plasmid construction The sequences of the primers used in this study are shown in Table 2. To construct the pVK9::PmsrA-CgynfM plasmid, the *msrA* promoter was amplified from chromosomal DNA of *C. glutamicum* AJ13869 (26) using the PmsrA-F and PmsrA-R primers, and PrimeSTAR Max DNA Polymerase (Takara Bio, Shiga, Japan). Bacterial chromosomal DNA was extracted using the PurElute Bacterial Genomic Kit (EdgeBio, Gaithersburg, MD, USA). The *CgynfM* gene was amplified from the chromosomal DNA of *C. glutamicum* AJ13869 with the ynfM-F/ynfM-R primer set. Next, the amplified DNA fragments were inserted into *Bam*HI- and *Pst*I-digested plasmid pVK9 (27) using the In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan). pVK9 is an *E. coli*–*C. glutamicum* shuttle vector obtained by integration of the *ori* region of plasmid pHM1519 (28) into plasmid pHSG299 (Takara Bio).

Construction of mutant strains The pBS4S::ΔsdhA plasmid was constructed to disrupt the *sdhA* gene. The N-terminal fragment of *sdhA* was amplified using chromosomal DNA from *C. glutamicum* AJ13689 as a template and the DsdhA-1/DsdhA-2 primer pair, whereas the C-terminal fragment was amplified using the DsdhA-3/DsdhA-4 primer pair. Next, both fragments were inserted into the pBS4S plasmid after *Bam*HI and *Pst*I digestion, using the In-Fusion HD Cloning Kit (Takara Bio), yielding pBS4SΔ*sdhA*. pBS4SΔ*sdhA* was introduced into the *C. glutamicum* AJ13689 strain, and single-crossover chromosomal integrants were selected on CM-Dex medium containing 25 μg/ml kanamycin, at 31.5°C for approximately 30 h. After isolating single colonies, they were subcultured in nonselective CM-Dex liquid medium such that a second homologous recombination event excised the plasmid DNA. The culture was spread onto CM-Dex agar medium, in which glucose was replaced with 10% (w/v) sucrose in the absence of kanamycin, and grown at 31.5°C for approximately 30 h. Clones obtained in this manner included those in which the original *sdhA* gene was replaced by the mutant gene derived from pBS4SΔ*sdhA*, and those in which *sdhA* had reverted to the wild-type gene. Whether *sdhA* was of the mutant or wild type was easily established by PCR. This method was used to obtain an *sdhA* disruptant.

The pBS4S::ΔCgynfM plasmid was constructed to disrupt the *CgynfM* gene. The N-terminal fragment of *CgynfM* was amplified using chromosomal DNA from *C. glutamicum* AJ13689 as a template and the DynfM-1/DynfM-2 primer pair, whereas the C-terminal fragment was amplified with the DynfM-3/DynfM-4 primer pair. Next, both fragments were inserted into the pBS4S plasmid after *Bam*HI and *Pst*I digestion, using the In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan), to produce the pBS4SΔCgynfM plasmid. pBS4SΔCgynfM was introduced into the FKS14 bacterial strain, and single-crossover chromosomal integrants were selected on CM-Dex medium containing 25 μg/ml kanamycin, at 31.5°C for approximately 30 h. After isolating single colonies, they were subcultured in nonselective CM-Dex liquid medium such that a second homologous recombination event excised the plasmid DNA. The culture was spread onto CM-Dex agar medium without kanamycin, in which glucose was replaced with 10% (w/v) sucrose, and grown for approximately 30 h at 31.5°C. The clones obtained in this way included those in which the original *CgynfM* gene had been replaced by the mutant derived from pBS4SΔCgynfM, and those in which *CgynfM* had reverted to the wild-type gene. PCR was used to readily determine the wild-type or mutant nature of *CgynfM*. This method was used to obtain a *CgynfM* disruptant.

Succinate fermentation under aerobic conditions Bacteria were pre-cultured on CM-Dex plates at 31.5°C for 16 h. The cells were then inoculated into

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Antibiotic resistance[a]	Reference or source
Strain			
FKS14	*Corynebacterium glutamicum* AJ13869Δ*sdhA*	None	This work
FKS27	*Corynebacterium glutamicum* AJ13869Δ*sdhA*Δ*CgynfM*	None	This work
FKS15	*Corynebacterium glutamicum* AJ13869Δ*sdhA*/pVK9	Km	This work
FKS20	*Corynebacterium glutamicum* AJ13869Δ*sdhA*/pVK9::PmsrA-CgynfM	Km	This work
AJ110655	*Corynebacterium glutamicum* MJ233Δ*ldh*	None	18
FK948	*Corynebacterium glutamicum* AJ110655Δ*sucE1*	None	18
FK902	*Corynebacterium glutamicum* AJ110655/pVK9	Km	18
FKS3	*Corynebacterium glutamicum* FK948/pVK9	Km	18
FKS34	*Corynebacterium glutamicum* FK948/pVK9::PmsrA-CgynfM	Km	This work
FKS35	*Corynebacterium glutamicum* FK948/pVK9::PmsrA-sucE1	Km	18
FK15	*Corynebacterium glutamicum* FK948/pVK9::PmsrA-AeyjjP	Km	21
Plasmid			
pVK9	An *Escherichia coli* and *Corynebacterium glutamicum* shuttle vector	Km	26
pVK9::PmsrA-CgynfM	pVK9 containing the *Corynebacterium glutamicum CgynfM* gene under control of the *msrA* promoter	Km	This work
pVK9::PmsrA-sucE1	pVK9 containing the *Corynebacterium glutamicum sucE1* gene under control of the *msrA* promoter	Km	18
pVK9::PmsrA-AeyjjPB	pVK9 containing the *Enterobacter aerogenes AeyjjPB* gene under control of the *msrA* promoter	Km	21

[a] Km, kanamycin.

TABLE 2. Sequences of primers used in this study.

Primer	Sequence (5′–3′)
PmsrA-F	CCAAGCTTGCATGCCATTTGCGCCTGCAACGTAGGTTG
PmsrA-R	AACAGGAATGTTCCTTTCGAAAA
CyjjPB-F	AGGAACATTCCTGTTATGCAAACTGAGCAACAGCG
CyjjPB-R	CGGTACCCGGGGATCGGAAGACATACTGTTTCTCA
CyjjP-R	CGGTACCCGGGGATCGGAACGTTGAACACCATCGC
CyjjB-F	AGGAACATTCCTGTTATGGGTGTGATCGAATTTCTG
yjjP-F	CCAAGCTTGCATGCCCATTAAAAAACGTGCCCGG
yjjPB-R	CGGTACCCGGGGATCACGTCCGGGGTCAAAACTCTG
yjjP-R	CGGTACCCGGGGATCAACGTTGAACACCATCGCAAAG
yjjB-F	CTACGCGGATGGGTGTGATC
Ptac-F	GGCCAGTGCCAAGCTCCCCCTGTGGCAAATTAATC
Ptac-R	CACCCATCCGCGTAGTCCTGTGTGAAATTGTTATC
Δ*sdhA-attL*	GCATATGTATGACACCGTCAAAGGTTCCGACTACATCGGTGACCAGGACGTGAAGCCTGCTTTTTTATACTAAGTTGGCA
Δ*sdhA-attR*	TCCAGCTCAAGGCACTCAATACGCTGTGTATTGAAGTCAGGTGAGCGGTCCGCTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ppc-F	TCAGGTGTGTTTAAAGCTGTTCTGCTGGGCAATACCCTGCAGTTTCGGGTCGCTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ppc-R	GTGAAGGATACAGGGCTATCAAACGATAAGATGGGGTGTCTGGGGTAATTGAAGCCTGCTTTTTTATACTAAGTTGGCA
Ptac-sucCD-F	TTACTGCTGCTGTGCAGACTGAATCGCAGTCAGCGCGATGGTGTAGACGACGCTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-sucCD-R	CCGCCATATGAACGGCGGGTTAAAATATTTACAACTTAGCAATCAACCATGAAGCCTGCTTTTTTATACTAAGTTGGCA
Ptac-ybjL-F	TTACCTTAGCCAGTTTGTTTTCGCCAGTTCGATCACTTCATCACCGCGTCCGCTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ybjL-R	ACACTAGTAAAATATATTGTTACTTTACTATCGTTTAGGTGCGCTGAATTGAAGCCTGCTTTTTTATACTAAGTTGGCA

test tubes containing 5 mL succinate fermentation medium [60 g L^{-1} glucose, 68 mg L^{-1} soybean protein hydrolysate (as total nitrogen), 6 g L^{-1} $(NH_4)_2SO_4 \cdot 7H_2O$, 0.54 g L^{-1} KH_2PO_4, 12 mg L^{-1} $MnSO_4 \cdot 5H_2O$, 12 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 120 μg L^{-1} biotin, and 120 μg L^{-1} thiamine·HCl ($VB_1 \cdot HCl$)] and 50 g L^{-1} precipitated $MgCO_3$, after which they were sterilized by dry heat at 180°C for 3 h (Nacalai Tesque, Kyoto, Japan). Following inoculation, succinate fermentation was performed at 31.5°C with a rotation speed of 120 rpm for 20 h.

Succinate fermentation under anaerobic conditions To examine succinate production in *C. glutamicum* under anaerobic conditions, we employed a convenient evaluation system involving 1.5-mL microcentrifuge tubes (20,27). Cells were pre-cultured on CM-Dex plates and then inoculated into 20 mL of SA1 medium [20 g L^{-1} glucose, 1.4 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 4 g L^{-1} urea, 0.02 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.02 g L^{-1} $MnSO_4 \cdot 7H_2O$, 200 μg L^{-1} biotin, 200 μg L^{-1} VB1·HCl, 1 g L^{-1} yeast extract, and 1 g L^{-1} casamino acids] and shaken in a test tube at 31.5°C for 20 h under aerobic conditions. Next, 700 μL of the pre-culture was collected and immediately mixed with 700 μL of SA2 medium [final concentration per liter: 100 g glucose, 15 g sodium sulfite, and 71.5 g $MgCO_3$ (sterilized separately)] in a microcentrifuge tube, followed by shaking at 32°C for 48 h under anaerobic conditions.

Metabolite analysis Organic acids that accumulated in the medium were analyzed by high-performance liquid chromatography on a CDD-10AD system (Shimadzu Co. Ltd., Kyoto, Japan) using a suitable dilution, as described in our previous report (18). Glucose concentrations were analyzed using an AS-310 Biotech Analyzer (Sakura SI Co. Ltd., Tokyo, Japan). The optical density (OD) was measured at 620 nm with a U-2001 spectrometer (Hitachi Co. Ltd., Tokyo, Japan). Broth containing $MgCO_3$ was diluted with 0.2 N HCl prior to measuring the OD_{620}.

Phylogenetic and multiple-alignment analyses of CgYnfM A multiple alignment was generated using the ClustalW multiple-sequence alignment web form (DNA Data Bank of Japan, http://clustalw.ddbj.nig.ac.jp) with sequences obtained from Transport DB 2.0 (http://www.membranetransport.org/transportDB2/index.html). The chosen matrix was BLOSUM; penalties were 10 (open gap), 0.1.

RESULTS

Effect of *CgynfM* expression and deletion on succinate production in *C. glutamicum* under aerobic conditions To confirm whether the putative membrane protein, CgYnfM, contributes to succinate production, the *CgynfM* gene was expressed or deleted in *C. glutamicum* strain AJ13869Δ*sdhA*, which is a succinate producer under aerobic conditions (12). The *CgynfM* gene was cloned into the pVK9 vector and expressed under the control of the *C. glutamicum msrA* (methionine sulfoxide reductase) promoter.

As shown in Fig. 1A, the succinate-production levels under aerobic growth conditions were 60 mM in strain FKS14 (AJ13869Δ*sdhA*), 62 mM in strain FKS27 (AJ13869Δ*sdhA*Δ*CgynfM*), and 66 mM in strain FKS15 (AJ13869Δ*sdhA*/pVK9). However, succinate production increased to 110 mM in strain FKS20 (AJ13869Δ*sdhA*/pVK9::PmsrA-CgynfM). Furthermore, *CgynfM* expression resulted in a decrease in residual glucose from 70 mM to undetectable levels, and the succinate yield (mol succinate·mol glucose^{-1}) increased from 26% to 33% (Fig. 1A). Succinate-specific productivity (mmol succinate·g cell mass^{-1}) also increased from 8.39 for strain FKS15 to 15 for strain FKS20 (Fig. 1B). In contrast, the succinate yield and succinate-specific productivity in FKS27 (AJ13869Δ*sdhA*Δ*CgynfM*) were comparable to that in FKS14 (AJ13869Δ*sdhA*).

High expression of the *CgynfM* gene led to significantly decreased production levels of acetate and pyruvate, which are major by-products, from 120 mM to 6.2 mM to undetected levels (Fig. 1C). The production levels of α-ketoglutarate (αKG), which is an intermediate in aerobic succinate biosynthesis, substantially increased following *CgynfM* expression from 3.2 mM to 120 mM (Fig. 1C), which exceeded the level of succinate production. Lactate production did not change regardless of *CgynfM* expression (data not shown). The production levels of succinate, malate, fumarate, αKG, pyruvate, and acetate were not changed by deleting *CgynfM*. These results show that *CgynfM* expression significantly affected succinate production in *C. glutamicum* under aerobic conditions.

CgynfM*-dependent succinate production in *C. glutamicum* lacking *sucE1 Previously, we identified a novel succinate transporter, SucE1, in *C. glutamicum* (18). Furthermore, we demonstrated that the *sucE1*-deletion mutant could not produce succinate because it lacked a succinate export carrier and that introducing the *sucE1* gene by plasmid transformation restored succinate production under anaerobic conditions (18). Therefore, we next examined whether succinate production could be restored by introducing *CgynfM* into *C. glutamicum* strain FK948, which cannot produce succinate due to disruption of the succinate transporter gene, *sucE1*. *CgynfM* was cloned into pVK9 and expressed under the control of the *C. glutamicum msrA* promoter. These plasmids were then introduced into strain FK948. Fig. 2 shows the results for succinate fermentation conducted in 1.5-mL microcentrifuge tubes under anaerobic conditions. Control strain FK902 (AJ110655/pVK9) produced 410 mM succinate, whereas FKS3 (AJ110655Δ*sucE1*/pVK9) produced only 9.5 mM succinate (Fig. 2A). However, succinate production was restored to 270 mM in strain FKS34 (AJ110655Δ*sucE1*/pVK9::PmsrA-CgynfM). Succinate-specific productivity (g succinate·g cell mass^{-1}) was 120 for strain FK902, 3.6 for strain FKS3, and 140 for FKS34 (Fig. 2B). These results demonstrate that the expression of *CgynfM* partly restored SucE1 function.

Furthermore, control strain FK902 (AJ110655/pVK9) produced 7.9 mM αKG, whereas FKS3 (AJ110655Δ*sucE1*/pVK9) produced 5.1 mM αKG (Fig. 2A). However, αKG production increased to 24 mM in strain FKS34 (AJ110655Δ*sucE1*/pVK9::PmsrA-CgynfM). The αKG-specific productivity (mmol succinate·g cell mass^{-1}) was 2.3 for strain FK902, 1.9 for strain FKS3, and 13 for FKS34 (Fig. 2B). These results suggest that *CgynfM* expression contributed to enhancing both succinate and αKG production under anaerobic conditions.

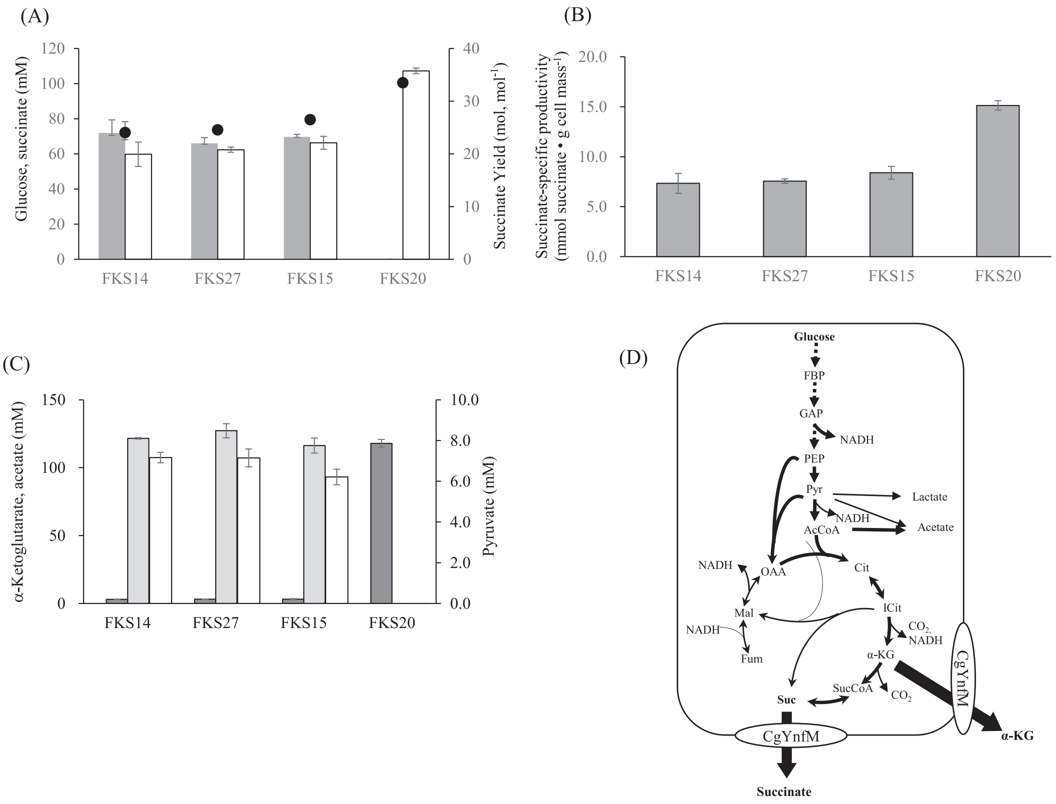

FIG. 1. Effect of *C. glutamicum CgynfM* expression on succinate production in *C. glutamicum* under aerobic conditions. Strain FKS14 was used as the host. *C. glutamicum* strains FKS14 (control), FKS27 (FKS14Δ*CgynfM*), FKS15 (FKS14/pVK9), and FKS20 (FKS14/pVK9::PmsrA-CgynfM) were used. Succinate production was performed in test tubes at 31.5°C with a rotation speed of 120 rpm for 20 h under aerobic conditions. (A) The glucose concentration (shaded columns), succinate concentration (open columns), and succinate yield (mol succinate·mol glucose^{-1}, closed circles) are shown. (B) Succinate specific productivity (mmol succinate·g cell mass^{-1}). (C) The αKG concentration (dark shaded columns), acetate concentration (light shaded columns), and pyruvate concentration (open columns) are shown. Data from three independent experiments are shown as the mean ± SE. (D) Pathways involved in succinate production under aerobic conditions in an SDH-deleted strain of *C. glutamicum*. The thin and thick arrows represent relatively smaller and larger fluxes, respectively.

Comparison of succinate transporters by succinate production in the *sucE1* deletion strain We previously found that not only SucE1, but also YjjPB from *E. aerogenes*, functions as a dicarboxylate transporter (20). Next, the substrate specificities of CgYnfM were compared to those of AeYjjPB and SucE1 by determining succinate production in the *sucE1*-deletion strain. We found that the *CgynfM*-expressing strain produced 270 mM succinate, 2.9 mM fumarate, 19 mM malate, and 24 mM αKG (Fig. 3). A *C. glutamicum* strain expressing *sucE1* produced 360 mM succinate, undetectable fumarate levels, 15 mM malate, and 8.9 mM αKG. In addition, a *C. glutamicum* strain expressing *AeyjjPB* produced 370 mM succinate, undetectable levels of fumarate, 4.1 mM malate, and 10 mM αKG. These results show that production levels of fumarate, malate, and αKG in the *CgynfM*-expressing strain were higher than those in *sucE1*-and *AeyjjPB*-expressing strains, suggesting that CgYnfM has broader substrate specificity for dicarboxylates. However, the production levels of lactate and pyruvate did not increase by expressing *CgynfM* under aerobic or anaerobic conditions (data not shown), suggesting that CgynfM has lower substrate specificity for lactate and pyruvate.

DISCUSSION

With succinate production in *C. glutamicum* under aerobic conditions, the by-product pyruvate remains as an issue, although the by-product acetate can be significantly reduced by deleting genes for all known acetate-producing pathways (*pta-acks*, *pqo*, and *cat*) and by expressing *acsA*, *gltA*, *ppc*, and *pyc* (16). To overcome this issue, we decreased both acetate and pyruvate by expressing *CgynfM* in an SDH-deletion strain without increasing the activities of acetate-producing pathways. Furthermore, this improvement increased the glucose-consumption rate and succinate-specific productivity. As a result, the succinate-production level increased from 66 mM to 107 mM, and the yield increased from 26% to 34% (Fig. 1A, B). To study the succinate–export activities of target proteins, we developed a new assay to examine whether expression of candidate genes encoding putative succinate transporters can restore succinate production in strains in which genes encoding known succinate exporters were disrupted (20,21). The assay indicated that CgYnfM partially restored succinate production (Fig. 2A), suggesting that CgYnfM could function as a succinate transporter. In this study, we demonstrated that expression of succinate transporters, not only SucE1 (18), YjjPB (20), and EaYjjPB (21), but also CgYnfM, has a large impact on aerobic succinate production in *C. glutamicum*. In the future, we will analyze differences in the properties of these transporters in detail using purified proteins to clarify which transporters are suitable for succinate production.

Although the αKG production level of the host strain (AJ13869Δ*sdhA*/pVK9) was only 3.0 mM, that of the strain expressing the *CgynfM* gene increased significantly to 110 mM, which was higher than the succinate production level. αKG is a by-product, but its production can be decreased by reducing the intracellular αKG concentration by enhancing the expression of αKG dehydrogenase (29), which converts αKG into succinyl-CoA.

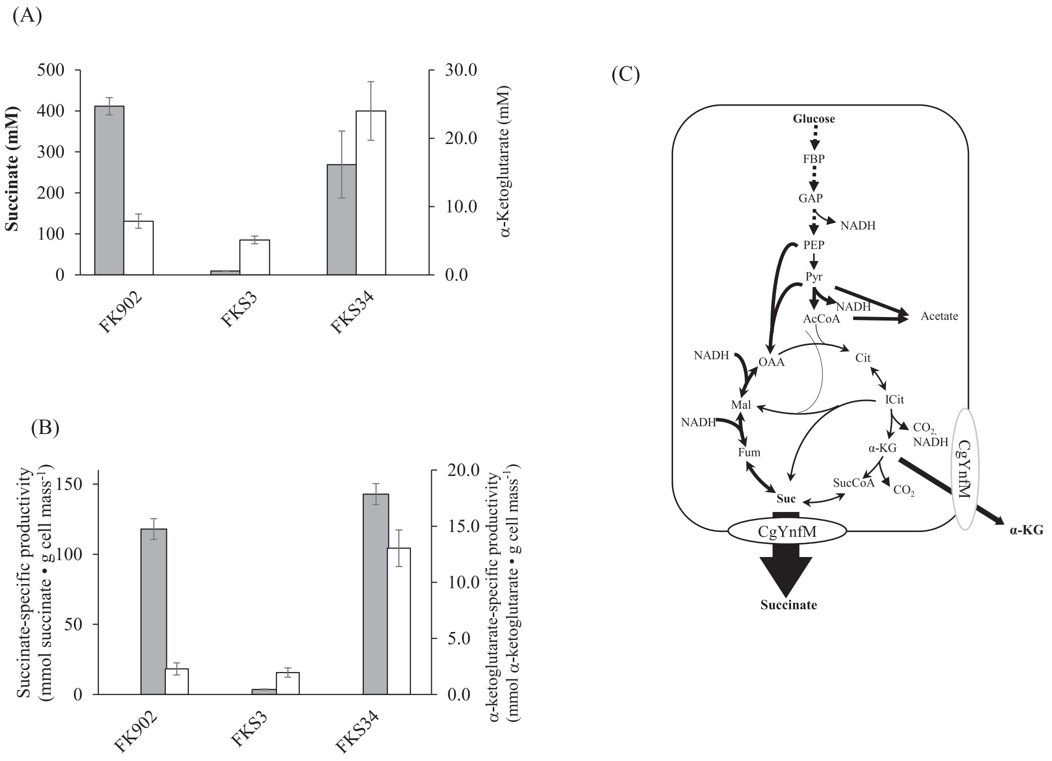

FIG. 2. *CgynfM*-dependent succinate production in the *sucE1*-deleted *C. glutamicum* strain. *C. glutamicum CgynfM* was introduced into *C. glutamicum* FK948, a strain in which the limiting step for succinate production is succinate export. The *C. glutamicum* FK902 (AJ110655/pVK9, control), FKS3 (AJ110655Δ*sucE1*/pVK9), and FKS34 (AJ110655Δ*sucE1*/pVK9::PmsrA-CgynfM) strains were used. Succinate production was performed in 1.5-mL microfuge tubes at 32°C with a rotation speed of 1400 rpm for 48 h under anaerobic conditions. (A) Succinate concentrations (shaded columns) and αKG concentrations (open columns) are shown. (B) The succinate-specific productivity (mmol succinate·g cell mass^{-1}; shaded columns) and αKG-specific productivity (mmol α-KG·g cell mass^{-1}; open columns) are shown. Data from three independent experiments are shown as the mean ± SE. (C) Pathways involved in succinate production under anaerobic conditions in an LDH-deleted *C. glutamicum* strain. The thin and thick arrows show relatively smaller and larger fluxes, respectively.

When performing the *in vivo* transport assays (Fig. 2), we found that αKG production increased from 5.1 mM to 21 mM, and both succinate- and αKG-specific productivities also increased (Fig. 2B). Taken together, these results suggest that CgYnfM has both succinate- and αKG-export activity. In an *ldh*-disrupted *C. glutamicum* strain, glucose was mainly metabolized to succinate via the reductive TCA cycle (theoretical yield: 1.71 mol succinate·mol glucose^{-1}) and partly via the oxidative TCA cycle to compensate for the reducing power required for the reductive TCA cycle (Fig. 2C) under anaerobic conditions. It was previously reported that the intracellular succinate and αKG concentrations of strain FK948 under microaerobic conditions were 8.1 mM and 0.80 mM, respectively (18). Based on these results, the higher flux of succinate production and intracellular succinate concentration (versus those of αKG) may explain why the succinate-production level of the FKS34 strain was 11-fold higher than that of αKG in *in vivo* transport assays (Fig. 2).

In the Transporter Classification Database (17), six bacterial transporters are listed as αKG transporters (Table 3). Two of these transporters (*E. coli* α-ketoglutarate permease [KGTP] and *Bacillus subtilis* CsbX) belong to the major facilitator superfamily (MFS). The αKG-transport activity of *E. coli* KGTP was demonstrated biochemically by performing transport assays using right-side-out membrane vesicles (30). Phylogenetic analysis of the amino acid sequences of all putative MFS transporters of *C. glutamicum* (Fig. 4A) revealed that CgYnfM is relatively close to the KGTP family transporters. Interestingly, both *E. coli* homologs of these transporters (CgYnfM and KGTP) were predicted to function as arabinose exporters (31). In the sequence alignment of *C. glutamicum* KGTPs and *C. glutamicum* CgYnfM (Fig. 4B), we identified conserved amino acid residues in two loops (connecting TM2 and TM3, and TM3 and TM4). These results indicated that CgYnfM and KGTPs share sequence and functional similarities with the MFS family.

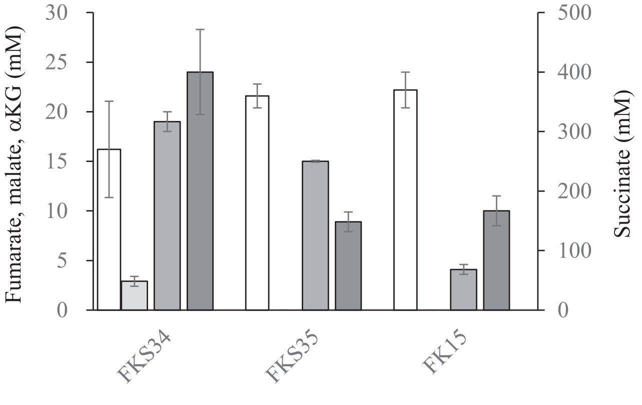

FIG. 3. Comparison of succinate-production levels after expressing different succinate transporters in the *sucE1*-deletion strain. *C. glutamicum CgynfM*, *sucE1*, and *E. aerogenes AeyjjPB* were introduced into *C. glutamicum* FK948, a strain in which the limiting step for succinate production is succinate export. Succinate production was performed in 1.5-mL microfuge tubes at 32°C with a rotation speed of 1400 rpm for 48 h under anaerobic conditions. (A) The concentrations of succinate (open columns), fumarate (the lightest shaded columns), malate (middle shaded columns), and αKG (the darkest shaded columns) are shown. Data from three independent experiments are shown as the mean ± SE.

TABLE 3. α-Ketoglutarate transporters listed in the Transporter Classification Database.

TC no.	Family	Accession no.	Protein	Species	Length	TMs[a]	Substrate
1.A.14.2.2	CaTA	P0AAC4	YbhL	*Escherichia coli*	234 aa	7	Acetate, serine, α-ketoglutarate, lactate, and succinate
2.A.1.6.2	MFS	P0AEX3	KGTP	*Escherichia coli*	432 aa	12	Arabinose, H$^+$, α-ketoglutarate
2.A.1.18.3	MFS	O05390	CsbX	*Bacillus subtilis*	435 aa	12	α-Ketoglutarate
2.A.47.1.11	DASS	Q2FFH9	SdcS	*Staphylococcus aureus*	520 aa	15	Succinate, malate, fumarate, aspartate, and α-ketoglutarate
2.A.47.1.13	DASS	Q65NC0	SdcL	*Bacillus licheniformis*	546 aa	14	Aspartate, α-ketoglutarate, and oxaloacetate
2.A.56.1.12	TRAP-T	Q8EJK8		*Shewanella oneidensis*	677 aa	20	α-Ketoglutarate

[a] TMs, transmembrane domains.

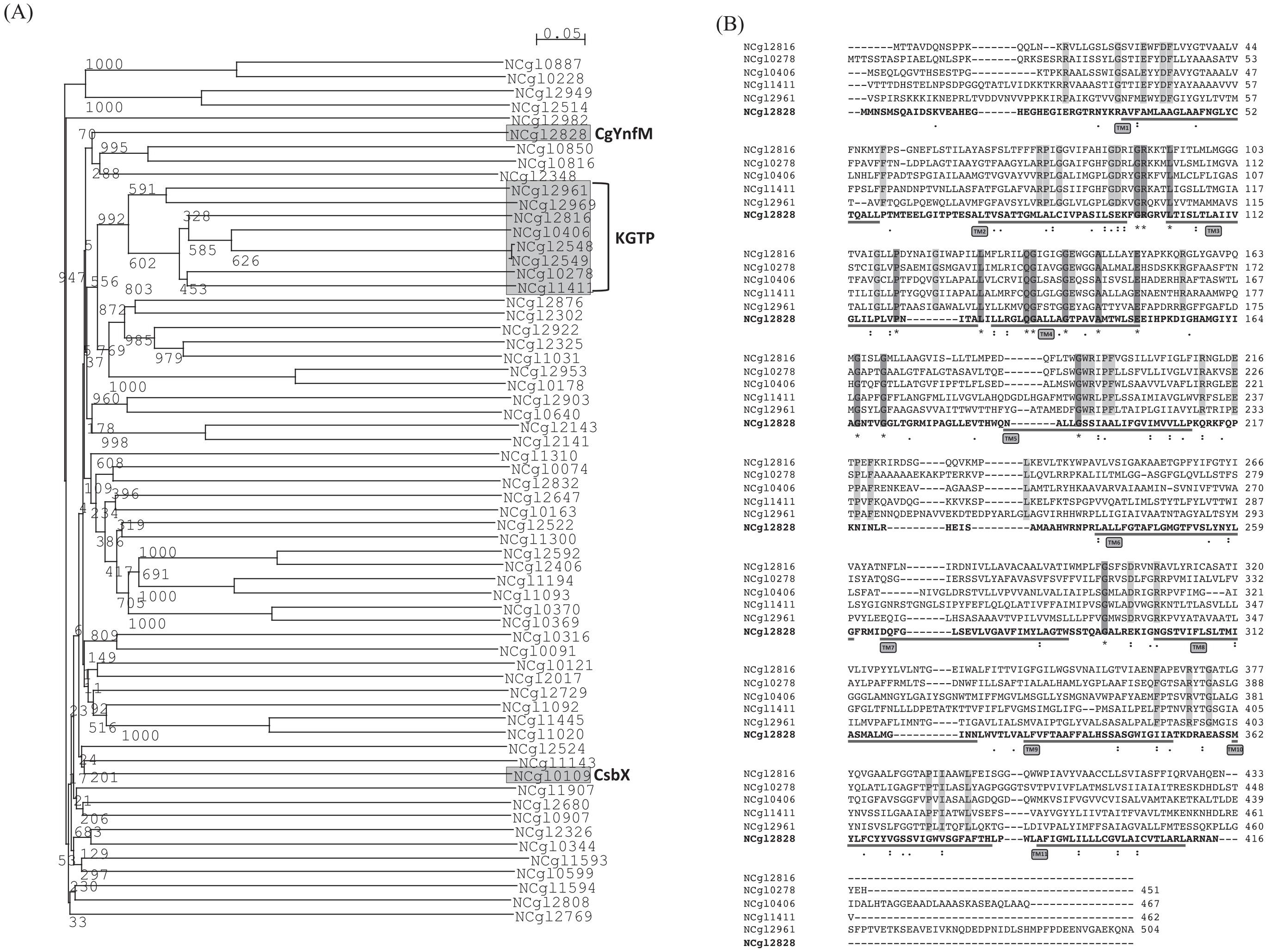

FIG. 4. Phylogenic analysis of amino acid sequences of *C. glutamicum* MFS transporters (A) and multiple-sequence alignment of KGTP homologs and CgynfM (B). (A) The amino acid sequences of *C. glutamicum* MFS transporters were aligned using ClustalW (2.1) (DNA Data Bank of Japan), and the phylogenetic tree was created using the alignment data with Njplot software (ver. 2.3). (B) Sequence alignments performed with ClustalW (2.1). The shadows identify similar residues. The asterisks (*) indicate positions with a single, fully conserved residue. The colons (:) indicate conservation between groups with strongly similar properties, based on a score of >0.5 obtained using the Gonnet PAM 250 matrix. The periods (.) indicate conservation between groups with weakly similar properties, based on a score of ≤0.5 obtained using the Gonnet PAM 250 matrix. The bold lines indicate transmembrane helices predicted by SOSUI (ver. 1.11).

After expressing known succinate transporters in the *sucE1*-deletion strain, we found that the production levels of fumarate, malate, and αKG in the *CgynfM*-expressing strain were higher than those in *sucE1*-and *AeyjjPB*-expressing strains, suggesting that CgYnfM has a broader substrate specificity for dicarboxylates (Fig. 3). αKG is of particular industrial interest due to its broad range of applications, namely, as a dietary supplement, a component of infusion solutions or wound-healing compounds, and as a building block for synthesizing heterocyclic molecules (32–34). Malic acid is also useful for producing pharmaceuticals, cosmetics, and acidulants in the food industry (35,36). CgYnfM may be effective for producing dicarboxylates, including αKG.

In conclusion, we performed a characterization of CgYnfM from *C. glutamicum* as a candidate succinate transporter. Expression of the *CgynfM* gene significantly increased aerobic succinate production in *C. glutamicum*, and our data suggested that the protein functions as a succinate and αKG transporter. CgYnfM was relatively close to the KGTP family transporters in all putative MFS transporters of *C. glutamicum*. Furthermore, CgYnfM seems to have a broader substrate specificity for dicarboxylates than other dicarboxylate transporters such as SucE and YjjPB, which is expected to influence its effectiveness for producing various dicarboxylates.

ACKNOWLEDGMENTS

We declare no conflict of interest.

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