Studies on Microbial Succinate Production

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ABBREVIATIONS

16srRNA-S	16s ribosomal RNA-S
NCgl2130-$[His]_6$	6x-histidine-tagged NCgl2130
ldhAΔ	*ldhA*-deletion mutant
LB medium	Luria-Bertani medium
TCA cycle	tricarboxylic acid cycle
CE-MS	capillary electrophoresis–mass spectrometry
Tris	tris(hydroxymethyl)aminomethane
DDM	n-dodecyl-β-D-maltoside
SD	standard deviation
SE	standard errors
$[His]_6$	histidine-tag
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
FBP	fructose-1,6-bisphosphate
NAD+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Km	kanamycin
Ap	ampicillin
Cm	chloramphenicol

Tet	tetracycline
Int/Xis	integrase/excisionase
λ Red	Lambda Red Recombineering System
OD	optical density
αKG	α–ketoglutarate
prpC	2-methylcitrate synthase gene
ppc	phosphoenolpyruvate carboxylase gene
msrA	methionine sulfoxide reductase gene
pta-ack	phosphotransacetylase-acetate kinase gene
pqo	pyruvate: quinone oxidoreductase gene
acsA	acetyl-CoA synthetase gene
gltA	citrate synthase gene
pyc	pyruvate carboxylase gene

GENERAL INTRODUCTION

Organic acids have long been used as raw materials in the food, chemical, and pharmaceutical industries. In recent times, ethyl lactate and diethyl succinate, which are esters of lactic acid or succinic acid (succinate), respectively, have become a focus of attention because they are environmentally benign solvents (1). Moreover, there is a growing market for lactate and succinate polymers as alternative raw materials for 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 anaerobes such as *Anaerobiospirillum succiniciproducens* (4,5), *Actinobacillus succinogenes* (6), and *Mannheimia succiniciproducens* (7). In the absence of oxygen and exogenous electron acceptors, the facultative anaerobes *Escherichia coli* and *Corynebacterium glutamicum* have been shown to produce succinate from sugars via the reductive TCA cycle (8–11). *Corynebacterium glutamicum*, which was originally isolated as an L-glutamate-producing bacterium (12), and has been used for the industrial production of various amino and nucleic acids (13,14), is a facultative anaerobe (15,16). In the absence of oxygen, if exogenous electron acceptors are not available, coryneform bacteria can produce succinate from sugars via the reductive TCA cycle (17,18).

Although 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 (19,20). Aerobic cell culture conditions were reported to have advantages over anaerobic conditions, such as higher biomass generation, and faster carbon throughput and product formation (20, 21). 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 (19, 21). 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 (22). *C. glutamicum* lacking the SDH also produced high amounts of acetate as a by-product (20). 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 (22).

On the other hand, it is known that exporters are important for microbial production (23-27). However, no succinate exporter has yet been identified, although succinate importers, such as DccT and DctA, have been found in *C. glutamicum* (28-30). Among the known succinate exporters, only the dicarboxylate uptake (Dcu) carriers DcuA–C in *E. coli* have been characterized so far (31-36). DcuAB carriers are used for electroneutral fumarate:succinate antiport, which is essential for anaerobic fumarate respiration, although the uptake and efflux of C4-dicarboxylate can be catalyzed as well (32,33). DcuC carriers are capable of the same transport activities, but their main function seems to be succinate efflux in *E.coli* (35,36). However, a search of the *C. glutamicum* genome made using the Basic Local Alignment Search Tool (BLAST) service revealed no homologs of Dcu transporters (such as DcuA, DcuB, DcuC, and DcuD). The author thus speculated that *C. glutamicum* also might have novel succinate exporters that differ from the Dcu-type carriers.

Lowering the pH of microbial cultures is considered a feasible approach to reducing the total costs of succinate production by limiting the use of alkali and acid in

the fermentation and recovery processes (37,38). Although anaerobic succinate production by *E. coli*, *C. glutamicum*, *Actinobacillus succinogenes*, and *Anaerobiospirillum succiniciproducens* has been studied at pH values ranging from 6.0 to 7.5 (39-41), few studies have focused on the effect of weakly acidic pH (<6.0) on succinate production in bacteria. This is because these bacteria are sensitive to acidic stress and are unable to grow and assimilate carbon-sources under acidic conditions (42,43). Previously, it has shown that *Enterobacter aerogenes* strain G243, a facultative anaerobe, is resistant to acidic stress under aerobic and anaerobic conditions (44-46). Furthermore, it has shown that this strain is a good producer of succinate from glucose via the reductive TCA cycle under anaerobic and weakly acidic conditions (44-46). However, no succinate exporter has yet been identified in *E. aerogenes* G243.

In this study, I studied some screens to identify succinate transporters in *C. glutamicum*, *E.coli*, and *E. aerogenes*, and examined the effect of them on microbial succinate production under aerobic and anaerobic condition.

References

1. **Zwicker, N., Theobald, U., Zahner, H., Fiedler, H.P.,:** Optimization of fermentation conditions for the production of ethylene-diamine-disuccinic acid by *Amycolatopsis orientalis*. J. Ind. Microbiol. Biotechnol. **19**, 280–285 (1997)
2. **Flieger, M., Kantorova, M., Prell, A., Rezanka, T., Votruba, J.,:** Biodegradable plastics from renewable sources. Folia Microbiol. **48**, 27–44 (2003)
3. **Zeikus, J.G., Jain, M.K., Elankovan, P.,:** Biotechnology of succinic acid production and markets for derived industrial products. Appl. Microbiol. Biotechnol. **51**, 542–552 (1999)
4. **Guettler, M.V., Jain, M.K.,:** Method for making succinic acid, *Anaerobiospirillum succiniciproducens* variants for use in process and methods for obtaining variants. U.S. patent 5521075 (1996)
5. **Lee, P.C., Lee, W.G., Kwon, S., Lee, S.Y., Chang, H.N.,:** Batch and continuous cultivation of *Anaerobiospirillum succiniciproducens* for the production of succinic acid from whey. Appl. Microbiol. Biotechnol. **54**, 23–27 (2000)
6. **Guettler, M.V., Rumler, D., Jain, M.K.,:** *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen. Int. J. Syst. Evol. Microbiol. **49**, 207–216 (1999)
7. **Lee, P.C., Lee, S.Y., Hong, S.H., Chang, H.N.,:** Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl. Microbiol. Biotechnol. **58**, 663–668 (2002)
8. **Dobrogosz, W. J.:** Altered end-product patterns and catabolite repression in *Escherichia coli*, J. Bacteriol., **91**, 2263–2269 (1996).

9. **Thakker, C., Martínez, I., San, K. Y., and Bennett, G. N.:** Succinate production in *Escherichia coli*, Biotechnol. J., **7**, 213–224 (2012).
10. **Okino, S., Inui, M., and Yukawa, H.:** Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation, Appl. Microbiol. Biotechnol., **68**, 475–480 (2005).
11. **Nianqing, Z., Huihua, X., Jiangang, Y., Xueming, Z., and Tao, C.:** Improved succinate production in *Corynebacterium glutamicum* by engineering glyoxylate pathway and succinate export system, Biotechnol. Lett., **36**, 553–560 (2014).
12. **Udaka, S.,** Screening method for microorganisms accumulating metabolites and its use in the isolation of *Micrococcus glutamicus*. J. Bacteriol. **79**, 754–755 (1960)
13. **Kinoshita, S., Udaka, S., Shimono, M.,:** Studies on the amino acid fermentation. Part1. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. **3**, 193–205 (1957)
14. **Shiratsuchi, M., Kuronuma, H., Kawahara, Y., Yoshihara, Y., Miwa, H., Nakamori, S.,:** Simultaneous and high fermentative production of L-lysine and L-glutamic acid using a strain of *Brevibacterium lactofermentum*. Biosci. Biotechnol. Biochem. **59**, 83–86 (1995)
15. **Nishimura, T., Vertès, A.A., Shinoda, Y., Inui, M., Yukawa, H.,:** Anaerobic growth of *Corynebacterium glutamicum* using nitrate as a terminal electron acceptor. Appl. Microbiol. Biotechnol. **75**, 889–897 (2007)
16. **Takeno, S., Ohnishi, J., Komatsu, T., Masaki, T., Sen, K., Ikeda, M.,:** Anaerobic growth and potential for amino acid production by nitrate respiration in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **75**, 1173–1182 (2007)
17. **Inui, M., Murakami, S., Okino, S., Kawaguchi, H., Vertès, A.A., Yukawa, H.,:**

Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J. Mol. Microbiol. Biotechnol. **7**, 182–196 (2004)

18. **Okino, S., Inui, M., Yukawa, H.,:** Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation. Appl. Microbiol. Biotechnol. **68**, 475–480 (2005)
19. **Lin, H., Bennett, G.N., and San, K.Y.:** Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield, Metab. Eng., **7**, 116–127 (2005).
20. **Litsanov, B., Kabus, A., Brocker, M., and Bott, M.:** Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*, Microb. Biotechnol., **5**, 116–128 (2012).
21. **Thakker, C., San, K.Y., and Bennett, G.N.:** Production of succinic acid by engineered *E. coli* strains using soybean carbohydrates as feedstock under aerobic fermentation conditions, Bioresour. Technol., **130**, 398–405 (2013).
22. **Zhu, N., Xia, H., Wang, Z., Zhao, X., and Chen, T.:** Engineering of acetate recycling and citrate synthase to improve aerobic succinate production in *Corynebacterium glutamicum*, PLoS ONE, **8**, e60659 (2013).
23. **Droshenko V., Airich L., Vitushkina M., Kolokolova A., Livshits V., and Mashko S.,:** YddG from *Escherichia coli* promotes export of aromatic amino acids, FEMS Microbiol Lett **275**, 312-318 (2007)
24. **Xu M., Rao Z., Yang J., Dou W., and Xu Z.,:** The Effect of a LYSE Exporter Overexpression on L-Arginine Production in *Corynebacterium crenatum*, Curr Microbiol. **67**, 271-278 (2013)

25. **Takumi K. and Nonaka G.,:** Bacterial Cysteine-Inducible Cysteine Resistance Systems, J Bacteriol, 198, 1384-1392 (2016)
26. **Kruse D., Krämer R., Eggeling L., Rieping M., Pfefferle W., Tchieu J.H., Chung Y.J., Saier Jr M.H., and Burkovski A.**,: Influence of threonine exporters on threonine production in *Escherichia coli*, **59**, 205-210 (2002)
27. **Liu Q., Liang Y., Zhang Y., Shang X., Liu S., Wen J. and Wen T.,:** YjeH Is a Novel Exporter of L-Methionine and Branched-Chain Amino Acids in *Escherichia coli*, Appl Environ Microbiol, **81**, 7753-7766 (2015)
28. **Teramoto, H., Shirai, T., Inui, M., Yukawa, H.,:** Identification of a gene encoding a transporter essential for utilization of C4 dicarboxylates in *Corynebacterium glutamicum*. Appl. Environ. Microbiol, **74**, 5290–5296 (2008)
29. **Youn, J.W., Jolkver, E., Kramer, R., Marin, K., Wendisch, V. F.,:** Identification and characterization of the dicarboxylate uptake system DccT in *Corynebacterium glutamicum*, J. Bacteriol, **190**, 6458–6466 (2008)
30. **Youn, J.W., Jolkver, E., Kramer, R., Marin, K., Wendisch, V.F.**,: Characterization of the dicarboxylate transporter DctA in *Corynebacterium glutamicum*. J Bacteriol, **191**, 5480–5488 (2009)
31. **Engel, P., Krämer, R., Unden, G.,:** Anaerobic fumarate transport in *Escherichia coli* by an fnr-dependent dicarboxylate uptake system which is different from the aerobic dicarboxylate uptake system, J. Bacteriol, **174**, 5533–5539 (1992)
32. **Engel, P., Krämer, R., Unden, G.,:** Transport of C4-dicarboxylates by anaerobically grown *Escherichia coli*. Energetics and mechanism of exchange, uptake and efflux, Eur. J. Biochem, **222**, 605–614 (1994)
33. **Six, S., Andrews, S.C., Unden, G., Guest, J.R.,:** *Escherichia coli* Possesses Two

Homologous Anaerobic C_4-Dicarboxylate Membrane Transporters (DcuA and DcuB) Distinct from the Aerobic Dicarboxylate Transport System (Dct), J. Bacteriol, **176**, 6470–6478 (1994)

34. **Unden, G., Bongaerts, J.,**: Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors, Biochim. Biophys. Acta, **1320**, 217–234 (1997)
35. **Zientz, E., Janausch, I.G., Six, S., Unden, G.,**: Functioning of DcuC as the C4-Dicarboxylate Carrier during Glucose Fermentation by *Escherichia coli*, J. Bacteriol., **181**, 3716–3720 (1999)
36. **Zientz, E., Six, S., Unden, G.,:** Identification of a Third Secondary Carrier (DcuC) for Anaerobic C_4-Dicarboxylate Transport in *Escherichia coli*: Roles of the Three Dcu Carriers in Uptake and Exchange, J. Bacteriol., **178**, 7241–7247 (1996)
37. **Cheng, K-K., Zhao, X-B., Zeng, J., Wu, R-C., Xu, Y-Z., Liu, D-H., and Zhang, J-A.:** Downstream processing of biotechnological produced succinic acid, Appl. Microbiol. Biotechnol., **95**, 841–850 (2012).
38. **Li, Q., Wang, D., Wu, Y., Li, W-L., Zhang, Y-J., Xing, J-M., and Su, Z-G.:** One step recovery of succinic acid from fermentation broths by crystallization, Sep. Purif. Technol., **72**, 294–300 (2010).
39. **Lu, S., Eiteman, M. A., and Altman, E.:** pH and base counterion affect succinate production in dual-phase *Escherichia coli* fermentations, J. Ind. Microbiol. Biotechnol., **36**, 1101–1109 (2009).
40. **Samuelov, N. S., Lamed, R., Lowe, S., and Zeikus, J. G.:** Influence of CO(2)-HCO(3) levels and pH on growth, succinate production, and enzyme activities of *Anaerobiospirillum succiniciproducens*, Appl. Environ. Microbiol., **57**,

3013–3019 (1991).

41. **Van der Werf, M. J., Guettler, M. V., Jain, M. K., and Zeikus, J. G.:** Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus sp.* 130Z, Arch. Microbiol., **167**, 332–342 (1997).
42. **Foster, J. W.:** *Escherichia coli* acid resistance: tales of an amateur acidophile, Nat. Rev. Microbiol., **2**, 898–907 (2004).
43. **Warnecke, T. and Gill, R. T.:** Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications, Microb. Cell Fact., **4**, 25 (2005).
44. **Tajima, Y., Kaida, K., Hayakawa, A., Fukui, K., Nishio, Y., Hashiguchi, K., Fudou, R., Matsui, K., Usuda, Y., and Sode, K.:** Study of the role of anaerobic metabolism in succinate production by *Enterobacter aerogenes*, Appl. Microbiol. Biotechnol., **98**, 7803–7813 (2014).
45. **Tajima, Y., Yamamoto, Y., Fukui, K., Nishio, Y., Hashiguchi, K., Usuda, Y., and Sode, K.:** Effects of eliminating pyruvate node pathways, and co-expression of heterogeneous carboxylation enzymes, on succinate production by *Enterobacter aerogenes*, Appl. Environ. Microbiol., **81**, 929–937 (2015).
46. **Tajima, Y., Yamamoto, Y., Fukui, K., Nishio, Y., Hashiguchi, K., Usuda, Y., and Sode, K.:** Impact of an energy-conserving strategy on succinate production under weak acidic and anaerobic condition in *Enterobacter aerogenes*, Microb. Cell Fact., **14**, 80 (2015).

CHAPTER I

Identification of succinate exporter gene *sucE1* in *Corynebacterium glutamicum* important for succinate production

In the present study, I carried out a comprehensive transcriptional analysis of *C. glutamicum* grown under microaerobic conditions using DNA microarrays, and identified some genes with expression that was upregulated under microaerobic conditions. Among the microaerobic-inducible genes I identified *sucE1*, which encodes a novel membrane protein that is homologous to the aspartate:alanine exchanger (AAE) family transporters. Here, I discuss and characterize this novel succinate exporter of *C. glutamicum* based on genetic functional analysis of the *sucE1* gene and biochemical transport analysis of SucE1 reconstituted into liposomes.

Materials and methods

Bacterial strains, growth conditions, and plasmids. *C. glutamicum* MJ233 (1, 2) was used as the wild-type strain, which is the closest to R strain as a result of sequence comparison of *NCgl2130* gene. The following mutants were constructed: Δ*ldhA*, Δ*ldhA*Δ*NCgl2130*, Δ*ldhA*/pKV9, Δ*ldhA*Δ*NCgl2130*/pVK9, Δ*ldhA*/pKV9*NCgl2130*, Δ*ldhA*Δ*NCgl2130*/pVK9*NCgl2130*, and Δ*ldhA*Δ*NCgl2130*/pFK5091. *E. coli* JM109 (Takara Bio, Otsu, Japan) and SCS110 (Agilent Technologies, CA, USA) were used for constructing and propagating plasmids. *E. coli* and *C. glutamicum* cells were cultured at 37 °C on Luria-Bertani (LB) medium

(3) and at 31.5 °C on CM-Dex medium (Nakamura et al., 2007), respectively. Plasmid pBS4S was used as a non-replicating integration vector (4). The final strains and plasmids studied are listed in Table 1.

Table 1. Strains and plasmids

Strains and plasmids	***Relevant characteristics***	***Reference or source***
Strains		
Wild type	*C. glutamicum* MJ233	(1)
Δ*ldhA*	Wild-type derivative with in-frame deletion of *ldhA*	This study
Δ*ldhA*ΔNCgl2130	Δ*ldhA* derivative with additional in-frame deletion of *NCgl2130*	This study
Plasmids		
pVK9	A shuttle vector of *E. coli* and *C. glutamicum*	(5) (6)
pVK9NCgl2130	pVK9 containing *NCgl2130* ORF	This study
pVK9::Ptuf-NCgl2130	pVK9 containing *NCgl2130* ORF with promoter replaced by *tuf* promoter	This study
pFK5091	pVK9::Ptuf-NCgl2130 containing $(His)_6$ in C terminus of *NCgl2130*	This study

Construction of mutant strains. DNA manipulation, sequencing, and bacterial transformation were performed as described previously (7). To disrupt genes, plasmids were constructed by two successive rounds of the polymerase chain reaction (PCR), and the primers used in this study are shown in Table 2.

ldhA disruptant; to disrupt the *ldhA* gene encoding NAD-dependent L-lactate

dehydrogenase (LdhA), in the first round one half of the fragment was amplified using the chromosomal DNA of *C. glutamicum* MJ233 as a template and the primer pair ldh-1 and ldh-2, while the other half was amplified with the primer pair ldh-3 and ldh-4. In the second round, the resulting products were mixed in a 1:1 molar ratio, and subjected to PCR using the primer pair ldh-5 and ldh-6 (the *Sal*I sites of which are underlined in Table 2). The amplified product was digested with *Sal*I, and ligated into *Sal*I-digested pBS4S, giving pBS4SΔ*ldh*56. pBS4SΔ*ldh*56 was introduced into the MJ233 strain, and the single-crossover chromosomal integrants were selected on CM-Dex medium containing 50 μg/ml kanamycin, at 31.5 °C for about 30 h. After single-colony isolation of the candidates, the colonies 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, at 31.5 °C for about 30 h. The clones obtained in this way included those in which the original *ldhA* gene had been replaced by the mutant derived from pBS4SΔ*ldh*56, and those in which the *ldhA* gene had reverted to wild type. Whether the *ldhA* gene was of the mutant or wild type was easily established by PCR. This method was used to obtain an *ldhA* disruptant.

NCgl2130 disruptant; similarly, to disrupt *NCgl2130* gene, in the first round one half of the fragment was amplified using the chromosomal DNA of *C. glutamicum* MJ233 as a template and the primer pair sucE1-1 and sucE1-2, while the other half was amplified with the primer pair sucE1-3 and sucE1-4. In the second round, the resulting products were mixed in a 1:1 molar ratio, and subjected to PCR using the primer pair sucE1-5 and sucE1-6 (the *Bam*HI sites of which are underlined in Table 2). The amplified product was digested with *Bam*HI and ligated into *Bam*HI-digested pBS4S, giving

pBS4SΔNCgl2130. pBS4SΔNCgl2130 was introduced into the Δ*ldhA* strain, and *NCgl2130* disruptants were obtained in the same way as the *ldhA* disruptant.

NCgl2130 over-expressing strain; *NCgl2130* gene was amplified using the chromosomal DNA of *C. glutamicum* MJ233 with the primers sucE1-F and sucE1-R (the *Sse*8387I sites of which are underlined in Table 2). The amplified DNA was cut with *Sse*8387I, and inserted into the *Sse*8387I site in the plasmid pVK9 (6) to generate the plasmid pVK9*NCgl2130*. pVK9 is an *E. coli–C. glutamicum* shuttle vector obtained by integration of the *ori* region of plasmid pHM1519 (5) into plasmid pHSG299 (Takara Bio). pVK9*NCgl2130* was introduced into the Δ*ldhA or* Δ*ldhA*Δ*NCgl2130 strains*, and the Δ*ldhA*/pKV9*NCgl2130* or Δ*ldhA*Δ*NCgl2130*/pVK9*NCgl2130* were selected on CM-Dex medium containing 50 μg/ml kanamycin, at 31.5 °C for about 30 h.

Table 2. Primers

Primer	***Sequence (5′–3′)***
ldh-1	CACTGCACGGCCCTGCGAAC
ldh-2	CGCCAACTAGGCGCCAAAAATTCCTGATTTCCCTAACCGGAC
ldh-3	GTCCGGTTAGGGAAATCAGGAATTTTTGGCGCCTAGTTGGCG
ldh-4	TGTGGGCCTTCGGCGAGGAC
ldh-5	GAGTCGACCGCACCCCATTTTTCATA
ldh-6	TGGTCGACGTGAATGCTCGGCGGGATCC
sucE1-1	GGGGGATCCGTGCTCTTTCTACACCTCTT
sucE1-2	GCGCTTAAGGGGTCAATGCTCCATTCATGATCTCATGGAT
sucE1-3	ATCCATGAGATCATGAATGGAGCATTGACCCCTTAAGCGC
sucE1-4	GGGGGATCCTCCTGGGTTGGCTTACCGGT
sucE1-5	GGGGGATCCATTTTCGCGGTTTCCTCACA
sucE1-6	GGGGGATCCTGGATGCAGAGGAACGTGTG
sucE1-F	GGGCCTGCAG GACCAAGACC GCTGTTGCAG TGA
sucE1-R	GGGCCTGCAG GGTATTCACA CCAGCCCCAAT

Bam0480f	GGGGATCCAGATCGTTTAGATCCGAAGGA
MJ21300480r	TTTTCTACAAGGAAGCTCACTGTATGTCCTCCTGGACTTC
0480MJ2130f	GAAGTCCAGGAGGACATACAGTGAGCTTCCTTGTAGAAAA
MJ2130850r	CCAAGGTCAGTGCGTTGTTG
sucE1-ApaBI-F	CGGAGCAAACCTTGCCC
sucE1-CtermHis-Rnew2	CTGCGCTTAAGGGGTCAATGCTAGTGGTGGTGGT
	GGTGGTGGATAAGTAGGAACAACAACGTTTGG
sucE1-CtermHis-Fnew2	CCAAACGTTGTTGTTCCTACTTATCCACCACCACCACCACCACTA
	GCATTGACCCCTTAAGCGCAG
sucE1-*EcoO*109I-R	CCCTAAGGGCCTCAAATACACG
16srRNA-S	GGAGAAGAAGCACCGGCTAA
16srRNA-A	ACGCTCGCACCCTACGTATT
sucE1-F1	GGCGCGGCGACACA
sucE1-R1	CACGATCACCTAGCTGCAAAGT

Succinate fermentation. For succinate fermentation, the following media were used; SA1 medium (composition per liter: 20 g glucose, 1.4 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 4 g urea, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.02 g $MnSO_4 \cdot 7H_2O$, 200 μg biotin, 200 μg thiamin·HCl (VB1·HCl), 1 g yeast extract, and 1 g casamino acid), SA2 medium (final concentrations per liter: 100 g glucose, 15 g sodium sulfite, and 71.5g $MgCO_3$ [separately sterilized]), SA3 medium (composition per liter: 20 g glucose, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g H_3PO_4, 14 ml soybean protein hydrolysate [total nitrogen, 0.5g], 1 g $(NH_4)_2SO_4$, 40 mg $FeSO_4 \cdot 7H_2O$, 40 mg $MnSO_4 \cdot 7H_2O$, 1 mg VB_1·HCl, 3 mg biotin, and 0.05 g GD-113 [antiform], pH 6.0 [KOH]), and SA4 medium (composition per liter: 0.75 g $MgSO_4 \cdot 7H_2O$, 1 g H_3PO_4, 4.2 ml soybean protein hydrolysate [total nitrogen, 0.15 g], 30 mg $FeSO_4 \cdot 7H_2O$, 30 mg $MnSO_4 \cdot 7H_2O$, 1.5 mg VB_1·HCl, 1.5 mg biotin, and 0.075 g GD-113 [antiform], pH 6.5 [KOH]).

Eppendorf tube-scale fermentation; Firstly preculture for cell growth was performed

in which cells cultured on CM-Dex plates were inoculated into 20 ml SA1 medium, and shaken in a Sakaguchi flask at 31.5 °C for about 8 h under aerobic conditions. Then, main culture for succinate production was performed in which 700 μl preculture was collected and immediately mixed with 700 μl SA2 medium in an Eppendorf tube, followed by shaking culture at 31.5 °C for about 48 h under anaerobic conditions.

Succinate fermentation in a jar fermentor; Firstly preculture for cell growth was performed in which cells cultured on CM-Dex plates were inoculated into 400 ml SA3 medium, and cultured at 30 °C, pH was maintained at 6.6 with ammonia gas. Aeration was applied at 400 ml/min and agitation was controlled to keep the dissolved oxygen concentration above 5 %. When glucose was consumed, 160 ml of 450 g/L glucose and 1 g/L GD-113 were added to the medium at a rate of approximately 10 ml/h. Then main culture for the succinate fermentation, 100 ml aliquots of the preculture were inoculated into 200 ml SA4 medium. The main culture was started by the addition of 80 ml of 595 g/L glucose solution, after all of the oxygen was removed by nitrogen gas (N_2). The culture was performed at 34 °C, with agitation at 500 revolutions per minute (rpm), and the pH was maintained at 7.3 with $Mg(OH)_2$. Under the microaerobic conditions in which the dissolved oxygen level was too low to measure with an oxygen electrode, 30 ml/min carbon dioxide (CO_2) gas and 20 ml/min air were supplied to the reaction, whereas under the anaerobic conditions, 30 ml/min CO_2 gas and 20 ml/min N_2 gas were supplied.

Analysis of organic acids. The amounts of succinate, malate, acetate, and pyruvate that had accumulated in the medium were analyzed by high-performance liquid chromatography (HPLC) after the medium had been suitably diluted. A column obtained by connecting two pieces of Shim-pack SCR-102H (Simazu, Japan) in series

was used, and the sample was eluted at 40 °C with 5 mM p-toluene sulfonic acid. The eluent was neutralized with a 20 mM Bis-Tris aqueous solution containing 5 mM p-toluene sulfonic acid and 100 μM ethylenediaminetetraacetic acid (EDTA). The succinate, acetate, and pyruvate were each assessed by measuring the electric conductivity with the CDD-10AD system (Simazu).

Extraction of intracellular metabolites. Intracellular metabolites were extracted as described previously, with some modifications (8). Culture broths including approximately 10^9 cells were filtered using a 0.45-μm-pore-size filter, and washed twice with 10 ml cold Milli-Q water. The filters were immersed in 2 ml methanol for quenching including 5 μM of each of the internal standards, L-methionine sulfone, 3-aminopyrrolidine dihydrochloride (3-AP), 2-(*N*-morpholino) ethane sulfonic acid (MES), D-camphor-10-sulfonic-acid sodium salt (CSA), and 1,3,5-benzenetricarboxylic acid (trimesate). L-methionine sulfone and 3-AP were used as internal cationic standards. MES, CSA, and trimesate were used as internal anionic standards. The dishes were sonicated for 1 min to suspend the cells completely. Portions (1.6 ml) of the methanol cell suspensions were mixed with 1.6 ml chloroform and 640 μl Milli-Q water for removing lipid. After vortexing well, the mixture was centrifuged at 4,600 ×*g* and 4 °C for 5 min. Portions (1.5 ml) of the aqueous layers were then distributed to six icon Ultrafree-MC ultrafilter tips (Millipore Co., MA, USA) and centrifuged at 9,100 ×*g* and 4 °C for approximately 2 h for removing proteins. The filtered extracts were analyzed using capillary electrophoresis–mass spectrometry (CE–MS) by Human Metabolome Technologies Inc. (Yamagata, Japan). The cell volume used to calculate the intracellular metabolite concentration was 1.8 μl mg^{-1} dry cell weight (DCW) (8). The DCW was calculated, as described previously, from the optical density at 620 nm (OD_{620}) using the

following experimentally obtained formula: DCW = 22.707 × (OD_{620}) / 100 (g, L^{-1}) (4).

Expression, solubilization, and purification of NCgl2130-$[His]_6$. In order to purify NCgl2130, pFK5091 including carboxy (C) terminus-histidine-tagged NCgl2130 was constructed as follows. Firstly, pVK9::Ptuf-*NCgl2130*, which included *NCgl2130* controlled by the *tuf* promoter encoding elongation factor Tu and was used as a template for pFK5091 including carboxy (C) terminus-histidine-tagged NCgl2130, was constructed as follows. In the first round, one half of the fragment was amplified using the chromosomal DNA of *C. glutamicum* MJ233 as a template and the primer pair Bam0480f and MJ21300480r (the *Bam*HI sites of which are underlined in Table 2), while the other half was amplified with the primer pair 0480MJ2130f and MJ2130850r. In the second round, the resulting products were mixed in a 1:1 molar ratio, and subjected to PCR using the primer pair Bam0480f and MJ2130850r. The amplified product, which included the *tuf* promoter, the upper region of NCgl2130, and the *Bst*XI site in the upper region of *NCgl2130*, was digested with *Bam*HI and *Bst*XI, and then ligated into *Bam*HI and *Bst*XI-digested pVK9*NCgl2130*, giving pVK9::Ptuf-*NCgl2130*. Secondly, to construct pFK5091, in the first round, one half of the fragment was amplified using pVK9::Ptuf-*NCgl2130* as a template and the primer pair sucE1-ApaBI-F and sucE1-CtermHis-Rnew2, while the other half was amplified with the primer pair sucE1-CtermHis-Fnew2 and sucE1-*EcoO*109I-R (the *Apa*BI *and* EcoO109I sites of which are underlined in Table 2). In the second round, the resulting products were mixed in a 1:1 molar ratio, and subjected to PCR using the primer pair sucE1-*Apa*BI-F and sucE1-*EcoO*109I-R. The amplified product was digested with *Bst*API and *EcoO*109I, and then ligated into *Bst*API and *EcoO*109I-digested pVK9::Ptuf-*NCgl2130*, giving pFK5091.

The Δ*ldhA*Δ*NCgl2130*/pFK5091 strain cultured on CM-Dex plates was inoculated into 100 ml SA1 medium containing 25 g/L glucose, and shaken in a Sakaguchi flask at 31.5 °C for 16 h under aerobic conditions. All subsequent steps were performed at 4 °C. After collection by centrifugation, the harvested cells (2 g of wet cell weight [WCW]) were suspended in 10 ml buffer containing 20 mM Tris-Cl (pH 8.0), 1 mM β-mercaptoethanol, and 0.1 ml/g-WCW Protein Inhibitor Cocktail VII (Merck, Darmstadt, Germany). Cells were disrupted three times by a French press at 20,000 psi. The cell debris was twice removed by centrifugation at 8,400 ×*g* for 30 min, and the precipitate was discarded. Cytoplasmic proteins released by the supernatant were removed by centrifugation at 149,000 ×*g* for 30 min, and membrane ghosts were obtained. These were solubilized (9) for more than 2 h with a buffer containing 20 mM Tris-Cl (pH 8.0), 300 mM NaCl, 20 % glycerol, 0.5 mM β-mercaptoethanol, 2 % (w/v) n-dodecyl-β-D-maltoside (DDM), 200 mM sodium succinate (pH 7.0), and 1 % (v/v) protein inhibitor cocktail VII. After solubilization on a rotator for more than 2 h followed by centrifugation at 149,000 ×*g* to remove the insoluble fraction, NCgl2130–$(His)_6$ was purified from the supernatant with TALONspin™ columns (Clontech Laboratories, Inc., CA, USA).

Reconstitution and transport assay of purified NCgl2130-$(His)_6$. The solubilized membrane proteins were reconstituted in a final volume of 1 ml with 800 μl detergent extract (including 120 μg protein or elution buffer as a control), 130 μl bath-sonicated liposomes (5.9 mg *E. coli* phospholipid), 2 μl of 10 % DDM, and an appropriate volume of KPi buffer (50 mM KH_2PO_4, and 100 mM K_2SO_4, pH 7.0 [KOH]). After incubation for 20 min on ice, proteoliposomes (or control liposomes) were formed at room temperature by rapid injection of the mixture into 20 ml loading

buffer containing 100 mM potassium succinate, 50 mM KH_2PO_4, and 50 μM phenylmethylsulfonyl fluoride (PMSF) at pH 7.0 (maintained with KOH). The substrate-loaded proteoliposomes (or liposomes) were kept at room temperature for 20 min. To make the concentrated proteoliposome preparation, the substrate-loaded proteoliposomes (or liposomes) were collected by centrifugation at 145,000 ×*g* for 60 min, and 300 μl KPi buffer was added to the precipitate.

In order to perform an assay for succinate transport with the concentrated proteoliposomes loaded with 100 mM succinate, the resulting suspensions were diluted 15-fold from the concentrated preparation into an appropriate volume of KPi buffer. After 3 min of pre-incubation at 25 °C, [^{3}H]-succinate was added to give a final concentration of 0.1 mM. At various time points, 100 μl aliquots were removed from the reaction mixture for filtration using 0.22-μm-pore-size GSTF Millipore filters. This was followed three times by washing with 5 ml assay buffer, and the radioactivities trapped on the filters were then measured (9).

The amounts of the protein in the purification experiments and in the proteoliposomes, were quantitatively measured using a BCA kit (Pierce, Rockford, IL, USA). In all cases, dilutions of bovine serum albumin (BSA) were used as standards.

Sequence comparison and *in silico* protein characterization. The SucE1 hydropathy analyses were conducted using SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Sequence similarity analyses of SucE1 were conducted using the Kyoto Encyclopedia of Genes and Genomes Sequence Similarity DataBase (KEGG SSDB;

http://www.genome.jp/kegg/ssdb/). Clustal W (http://align.genome.jp/) was used for comparisons and to display the phylogenetic tree. Sequence similarities of SucE1 with sequences of the homolog from a variety of other bacterial was analyzed using GENETYX software ver.9.

DNA microarrays and reverse-transcription quantitative PCR (RT-qPCR). DNA microarrays; Cells were cultivated in 400 ml SA3 medium in a jar fermentor. When glucose was consumed, 160 ml of 450 g/L glucose and 1 g/L GD-113 were added to the medium at a rate of approximately 10 ml/h. Aeration was applied at 400 ml/min and agitation was controlled to keep the dissolved oxygen concentration above 5 %. The sample under aerobic conditions was taken just before the glucose was completely consumed. Immediately afterwards, an additional 30 ml of 450 g/L glucose and 1 g/L GD-113 were added to the medium, and the agitation and aeration were reduced to 400 rpm and 100 ml/min, respectively. Under these conditions, the dissolved oxygen level was close to 0 %. After 2 h, a sample was taken under the microaerobic conditions. Cells were cultured at 30 °C, and the pH was maintained at 6.6 with ammonia gas.

Total RNA, at a concentration of more than 1 μg/μl and a quantity of more than 40 μg, was prepared using an RNeasy kit (Qiagen, Hilden, Germany). DNA microarray analysis was carried out by the Genefrontier Corp. (Japan).

RT-qPCR; DNA was removed completely from the total RNA prepared by the RNeasy kit (Qiagen) using a TURBO DNA-free™ kit (Applied Biosystems, CA, USA). Total complementary DNA (cDNA) was then prepared by a Prime Script reagent kit (Takara Bio) using 100 ng DNA-free RNA as a template. RT-qPCR was performed using a 7500 real-time PCR system and a Power SYBR Green PCR Master Mix (both from Applied Biosystems). Specific primers (Table 2) were designed using the Primer

Express Software version 2.0 (Applied Biosystems). sucE1-F1 and sucE1-R1, and 16s ribosomal RNA-S (16srRNA-S) and 16srRNA-A, were used as *sucE1* and 16S rRNA amplifying primers, respectively. Each PCR reaction comprised 12.5 μl of 2 × Power SYBR Green PCR Master Mix, 0.4 μM forward primer, 0.4 μM reverse primer, and 10 ng total RNA in a total volume of 25 μl. The specificity of the amplicons was checked by RT-qPCR dissociation-curve analysis. The comparative threshold-cycle method (Applied Biosystems) was used to quantify the relative expression.

Results

Effect of *NCgl2130* deletion on succinate production. Previously, it was reported that aerobically grown *C. glutamicum* R strain cells produced organic acids, mainly lactate, succinate, and acetate, from glucose under anaerobic conditions (10), and that an *ldhA*-deletion mutant (*ldhAΔ*) produced mainly succinate and no lactate (11). I therefore postulated that the expression levels of genes related to succinate production in *ldhAΔ* would be higher than those in the wild type, and that both strains would contain some genes encoding succinate exporters. To identify the succinate-export system, I comparatively analyzed transcripts extracted from *C. glutamicum* MJ233Δ*ldhA* cells grown under aerobic (non-succinate-producing) and microaerobic (succinate-producing) conditions, and extracted those genes that were annotated as encoding membrane proteins and that showed more than twofold greater expression levels under microaerobic conditions than under aerobic conditions (data not shown). The *NCgl2130* gene (similar to *cg2425*) met both of these conditions, and RT-qPCR revealed its expression to be threefold greater under microaerobic conditions than under

aerobic conditions (Fig. 1). I subsequently examined the succinate production of a *NCgl2130*-overexpressing strain and a *NCgl2130* mutant at an Eppendorf-tube scale under anaerobic conditions, using sodium sulfite to remove oxygen completely. The results of the fermentation showed that the succinate accumulation was 174 mM for the Δ*ldhA*/pKV9 strain and 274 mM for the Δ*ldhA*/pKV9*NCgl2130* strain (Fig. 2). Succinate production was increased 1.5-fold by overexpression of the *NCgl2130* gene. By contrast, as expected, the Δ*ldhA*Δ*NCgl2130*/pVK9 strain did not produced succinate under these conditions. Furthermore, in the Δ*ldhA*Δ*NCgl2130*/pVK9*NCgl2130* strain, which was obtained by plasmid complementation of the *NCgl2130* gene with the Δ*ldhA*Δ*NCgl2130* strain, the succinate production was restored to the same level as seen in the latter; this implied that the *NCgl2130* carried on the pVK9*NCgl2130* plasmid was expressed at a much higher level than the authentic *NCgl2130* gene carried on the chromosome. These results revealed that *NCgl2130*, the transcription of which was increased under microaerobic conditions, was essential for succinate production, and that its overexpression improved fermentative succinate production under anaerobic conditions. This gene was annotated as a predicted permease, although little was known about its function, with the exception that its expression was decreased in a *sugR*-deletion mutant in which LdhA activity under aerobic and oxygen-deprived conditions was increased, and L-lactate production under oxygen-deprived conditions was enhanced threefold (12). I therefore investigated whether it encoded a novel succinate transporter that catalyzed succinate excretion from cells.

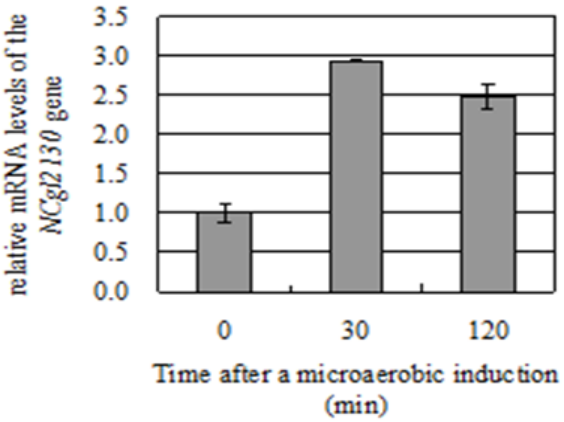

Fig. 1. Effect of microaerobic induction on *NCgl2130* expression. The levels of *NCgl2130* messenger RNA in the Δ*ldhA* strain during microaerobic induction (at 0, 30, and 120 min) were analyzed by RT-qPCR, and compared with those immediately after the shift to microaerobic conditions. Mean values from three independent cultures are shown with SDs.

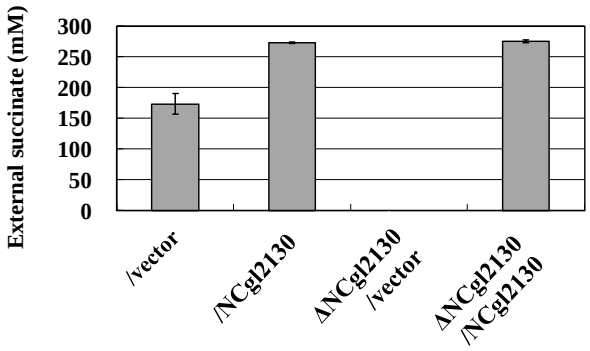

Fig. 2. Effects of disruption and amplification of *NCgl2130* on succinate production under anaerobic conditions. Δ*ldhA* was used as a host strain. Succinate production was performed by an Eppendorf-tube-scale fermentation under the conditions described in the Materials and Methods. The graphs show growth (○), extracellular residual glucose (empty columns) and succinate concentrations (gray columns). The data from three independent experiments are shown as means ± SEs.

Succinate production in a *NCgl2130*-disrupted mutant in jar fermentor under microaerobic and anaerobic conditions. In order to further confirm that *NCgl2130* is involved specifically in succinate production in *C. glutamicum*, I measured the succinate production of the Δ*ldhA* and Δ*ldhA*Δ*NCgl2130* strains using a jar fermentor under microaerobic and anaerobic conditions.

Under microaerobic conditions, initial values of biomass in both strains were the same, and cell growth almost ceased in both strains, since only small amount of oxygen was added. OD620nm of the Δ*ldhA* strain in 2h was similar to the Δ*ldhA*Δ*NCgl2130* strain (Δ*ldhA* strain; 59.0, Δ*ldhA*Δ*NCgl2130* strain; 54.7). Under this condition, the Δ*ldhA* strain produced 60 mM succinate, 30 mM acetate, and only small amounts of pyruvate and malate (Fig. 3 (A)). By contrast, the Δ*ldhA*Δ*NCgl2130* strain produced 40 mM succinate, 30 mM acetate, and similar amounts of pyruvate and malate to those produced by the Δ*ldhA* strain (Fig. 3 (B)). Hence, only the succinate accumulation by Δ*ldhA*Δ*NCgl2130* decreased (by approximately 30 %) compared with that by the Δ*ldhA* strain. The glucose consumption in 2 h for the Δ*ldhA*Δ*NCgl2130* strain was 80 % of that for the Δ*ldhA* strain (85 mM versus 110 mM; Fig. 3 (A), (B)). Under anaerobic conditions, in which cell growth of the two strains ceased, the Δ*ldhA* strain consumed 110 mM glucose, and produced 60 mM succinate and 20 mM acetate, in 2 h (Fig. 3 (A)). Pyruvate and malate productions were interrupted under anaerobic conditions. Interestingly, the Δ*ldhA*Δ*NCgl2130* strain consumed relatively little glucose under anaerobic conditions, and consequently produced relatively small amounts of organic acids (Fig. 3 (B)). As a result of succinate production in the Δ*ldhA*Δ*NCgl2130* strain in jar fermentor culture under microaerobic and anaerobic conditions, it was confirmed that NCgl2130 is involved specifically in succinate production in *C. glutamicum*.

Furthermore, the intracellular succinate concentrations of the Δ*ldhA* and Δ*ldhA*Δ*NCgl2130* strains under microaerobic conditions were 4.9 mM and 8.1 mM, respectively (Table 3); hence, the *NCgl2130* disruption caused the appoximately1.7-fold increase in the intracellular succinate concentration, although the extracellular succinate accumulation of the Δ*ldhA*Δ*NCgl2130* strain was only 70 % (41.5±0.8 mM) of that of the Δ*ldhA* strain (60.4±2.7mM) (Fig. 3).

These results suggest that the *NCgl2130* gene could have an important role in succinate production, possibly as a succinate exporter because of the fact that the putative amino acid sequence of NCgl2130 is predicted as a membrane protein by hydropathy analysis based on the prediction program SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/). Nevertheless, the Δ*ldhA*Δ*NCgl2130* strain continued to produce succinate under microaerobic conditions, implying that at least one succinate exporter other than *NCgl2130* was functional.

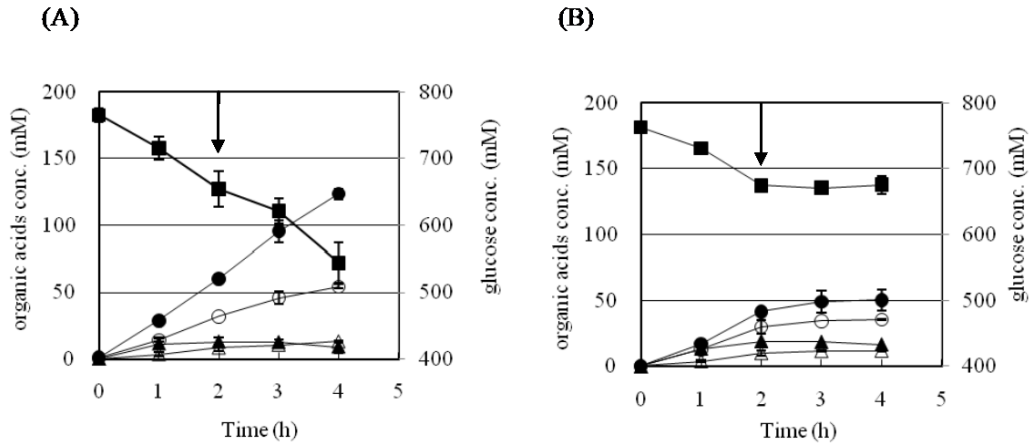

Fig. 3. Succinate production in jar fermentors under microaerobic and anaerobic conditions. Cells were grown on glucose under aerobic conditions at 30 °C, and the pH was maintained at 6.6 with ammonia gas. For the succinate fermentation, the cells were inoculated into succinate production medium. Fermentation at 34 °C with agitation at 500 rpm and pH maintained at 7.3 with $Mg(OH)_2$ was performed under microaerobic conditions for the first 2 h with 30 ml/min CO_2 gas and 20 ml/min air supplied to the reaction, and then under anaerobic conditions for the next 2 h with 30 ml/min CO_2 gas and 20 ml/min N_2 gas supplied to the reaction. (A) Δ*ldhA* strain. (B) Δ*ldhA*Δ*NCgl2130* strain. The graphs show the consumption of (■) glucose, (●) succinate, (○) acetate, (▲) pyruvate, and (△) malate, and the sampling point for metabolome analysis under microaerobic conditions is indicated by an arrow. The data from three independent experiments are shown as means ± SEs.

NCgl2130-$(His)_6$ purification, reconstitution, and transport assay. The decrease in succinate production and increase in the intracellular succinate concentration caused by the *NCgl2130* disruption implied that the putative NCgl2130 protein could be a succinate exporter. Hydropathy analysis suggested that NCgl2130 is a membrane protein, which supports the prediction based on the culture experiments. To examine whether NCgl2130 functioned as a succinate transporter, I purified the protein, reconstituted it into liposomes, and performed a counterflow experiment. I constructed the expression vector pFK5091, in which a 6x histidine tag was added to the C-terminus of NCgl2130 in pVK9::Ptuf-*NCgl2130*. The NCgl2130-$(His)_6$ expressed on pFK5091 was found to be functional *in vivo*, because the Δ*ldhA*Δ*NCgl2130*/pFK5091 strain produced a similar concentration of succinate to the Δ*ldhA*Δ*NCgl2130*/pVK9::Ptuf-*NCgl2130* strain during fermentation (data not shown). To purify NCgl2130, the membrane fractions of Δ*ldhA*Δ*NCgl2130*/pFK5091 cells containing NCgl2130-$(His)_6$ were solubilized with a buffer containing DDM, and subsequently subjected to purification using a TALONspin™ column. A Coomassie Brilliant Blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel was used to demonstrate the purification of NCgl2130-$(His)_6$ by the metal affinity chromatography (Fig. 4). To identify the protein, the band (indicated by an arrow in Fig. 4) was extracted from the SDS-polyacrylamide gel and digested with trypsin, and the enzymatically fragmented peptides were analyzed by nano-liquid chromatography/tandem MS (LC-MS/MS). The result indicated that the protein was NCgl2130 (data not shown).

The partially purified NCgl2130-$(His)_6$ was reconstituted into liposomes that were prepared from *E. coli* lipids. The transport function of the reconstituted NCgl2130-$(His)_6$ was assayed based on succinate counterflow activity. When

proteoliposomes were preloaded with nonradioactive succinate, the accumulation of [^{3}H]-succinate was observed with an exchange rate of 2.4 nmol min^{-1} mg protein^{-1} (Fig. 5). This result demonstrated that NCgl2130 was successfully reconstituted into the proteoliposomes in a functionally active form, and thus proven to be a succinate transporter. The author designated *NCgl2130* gene *sucE1*.

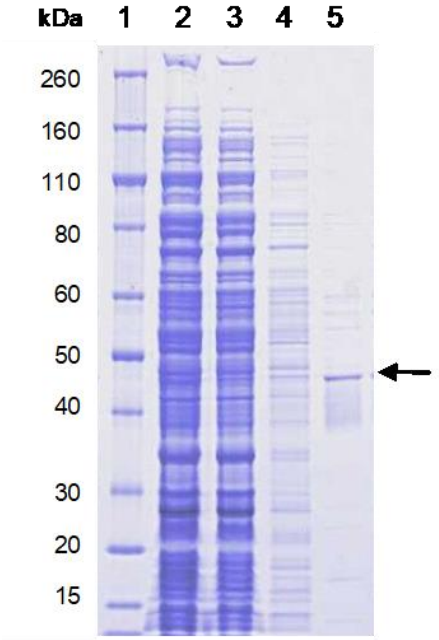

Fig. 4. Purification of NCgl2130. The protein compositions of fractions obtained during the purification of SucE1 were determined by SDS-polyacrylamide gel electrophoresis (PAGE). Lane 1, Novex Sharp Unstained protein marker (Invitrogen); lane 2, crude detergent extract; lane 3, flow-through fraction; lane 4, first-wash fraction; lane 5, 150 mM imidazole-eluted NCgl2130 fraction. An arrow indicates NCgl2130 protein. The positions of the molecular-mass standards are indicated.

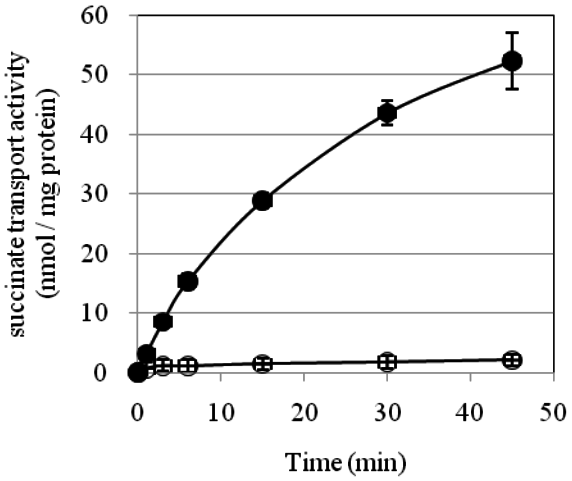

Fig. 5. Succinate counterflow in proteoliposome vesicles. Purified NCgl2130 was reconstituted into proteoliposomes loaded with 100 mM potassium succinate and 50 mM KH_2PO_4 (pH maintained at 7.0 with KOH). After collection, the proteoliposome was resuspended in KPi buffer, and then diluted 17-fold in the same buffer containing 100 mM [^{3}H]succinate. Transport was assayed as described in the Materials and Methods. The graph shows data for (●) proteoliposomes including NCgl2130 and (○) liposomes from three independent experiments (means ± SEs).

Hydropathy and sequence-similarity analyses of SucE1. According to the computational classification proposed by Saier (http://www.tcdb.org/) (13), AAE family transporters uniquely possess 10–12 putative transmembrane segments (TMSs) and a large hydrophilic cytoplasmic loop (between transmembrane domain 5 [TM5] and TM6) consisting of duplicated TrkA_C domains (13). SucE1 hydropathy and sequence similarity analyses predicted the presence of 9–10 TMSs, and duplicated TrkA_C domains between the amino (N)-terminal and carboxy (C)-terminal halves of the TMSs. Three different programs such as the SOSUI predicted the presence of 9 , 9, and 10 TMSs in SucE1, respectively. Although the three programs generated two different topology models (9 and 10 TMSs) of SucE1, all models showed the presence of conserved duplicated TrkA_C domains in a large hydrophilic loop between N-terminal and C-terminal halves of 9-10 TMs. At least, the predictions suggest that SucE1 belongs to the AAE family.

Next, I examined whether succinate producers, such as *E. coli* (14, 15), *A. succinogenes* 130Z (16), and *M. succiniciproducens* (17), had SucE1 homologs. A BLAST search using the SucE1 amino-acid sequence revealed that not only corynebacterial species but also succinate producers have two SucE1 homologs. The AspT found in lactic-acid bacteria is the prototype carrier of the AAE family, which takes up aspartate and exports alanine (18,19). The phylogenetic tree for the SucE1 homologs of the bacteria and the aspartate:alanine antiporter AspTs produced using Clustal W is shown in Fig. 6. All of the SucE1 and AspT homologs shown in the tree, except for MS0288 and MS0289 from *M. succiniciproducens*, possessed 9–12 putative TMSs and duplicated TrkA_C domains between the N-terminal and C-terminal halves of the TMSs. MS0288 and MS0289 corresponded to the N-terminal and C-terminal

halves of a typical AAE family member, and each possessed a single hydrophobic element (5–6 TMSs) and a single TrkA–C domain. The members fell into four clusters (I-IV), and SucE1 was classified into a clearly distant cluster (cluster I) from the cluster IV including AspT. The first two clades (cluster I, II) were sets of orthologous corynebacterial proteins (cluster I- SucE1, CE2102, and DIP0830; and cluster II - NCgl0565, CE595, and DIP0570). The third set of orthologous γ-proteobacterial proteins (Asuc0023, MS0288, MS0289, and YidE [b3685]) clustered loosely together with the two sets of orthologous corynebacterial proteins (cluster III). The fourth set of AspT homologs (cluster IV), which included YbjL (b0847) derived from *E. coli*, was distant from the cluster I including SucE1 homologs.

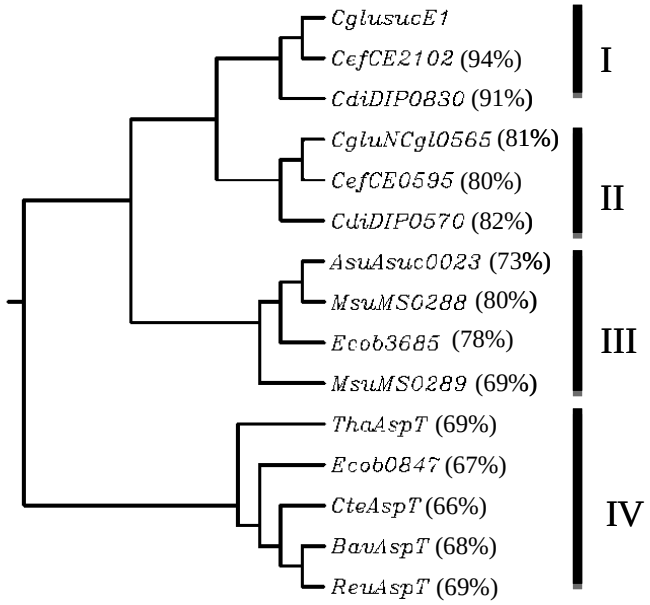

Fig. 6. Dendrogram of the deduced amino-acid sequence of the *C. glutamicum sucE1* gene product and the sequences of homologs from various other bacteria. All sequence data were obtained from the GenBank database using the following accession numbers: *C. glutamicum* cgl NCgl2130, **NP_601414**; *C. glutamicum* cgl NCgl0565, **NP_599826**; *C. efficiens* cef CE2102, **NP_738712**; *C. efficiens* cef CE0595, **NP_737205**; *C. diphtheriae* cdi DIP0570, **NP_938946**; *C. diphtheriae* cdi DIP0830, **NP_939194**; *E. coli* eco b3685, **NP_418140**; *E. coli* eco b0847, **NP_415368**; *M. succiniciproducens* msu MS0289, **YP_087481**; *M. succiniciproducens* msu MS0288, **YP_087480**, *A. succinogenes* Asu Asuc_0023, **YP_001343340**; *Tetragenococcus halophilus* ThaAspT, aspartate:alanine antiporter, **BAB92081**; *Comamonas testosteroni* CteAspT, aspartate:alanine antiporter, **BAC65228**; *Ralstonia eutropha* ReuAspT, putative aspartate:alanine antiporter, **YP_726110**; and *Bordetella avium* BavAspT, putative aspartate:alanine antiporter, **CAJ48975**. Sequence similarities with *C. glutamicum* SucE1 are shown after each name.

Metabolome analysis of *sucE1*-deletion mutant under microaerobic conditions. I showed that succinate production at the culture level was significantly decreased by the *sucE1* disruption as described above (Fig. 3).

The concentrations of intracellular metabolites comparatively measured in the Δ*ldhA*, and Δ*ldhA*Δ*sucE1* strains under microaerobic conditions are shown in Table 3. Notably, the glycolytic metabolite in the Embden-Meyerhof pathway (EMP) upstream of fructose-1,6-bisphosphate (FBP) accumulated while the numerous TCA intermediates, with the exception of succinate, declined with the deletion of *sucE1*. In the Δ*ldhA*Δ*sucE1* strain, similar to succinate, the intracellular levels of glucose-6-phosphate, fructose-6-phosphate, and FBP (3.9, 0.5, and 41.3 mM, respectively) were greater than those that accumulated in the Δ*ldhA* strain (by 1.8-fold, 1.7-fold, and 1.6-fold, respectively). Among the other metabolites, the concentration of citrate decreased by 55 % (from 2.0 mM to 0.9 mM), 2-oxoglutarate decreased by 30 % (from 1.2 mM to 0.8 mM), and malate decreased by 30 % (from 16.3 mM to 10.8 mM), although the succinate concentration was increased by the *sucE1* mutation (from 4.9 mM to 8.1 mM). Glyceraldehyde-3-phosphate and 1,3-bisphospho-glycerate were not analyzed, because their standard samples were not included in the system.

Table 3. Intracellular metabolite concentrations[a] under microaerobic conditions (mM)

	ΔldhA	***ΔldhAΔsucE1***
Glucose-6-phosphate	2.2±0.1	3.9±0.2
Fructose-6-phosphate	0.3±0.0	0.5±0.0
Fructose-1,6-diphosphate	26.3±0.8	41.3±2.1
Dihydroxyacetone phosphate	N.D.	N.D.
3-phosphoglycerate	3.1±0.2	3.1±0.1
Phosphoenolpyruvate	0.1±0.0	0.1±0.0
Pyruvate	N.D.	1.4±0.2
Acetyl-CoA	0.2±0.0	0.2±0.0
Malate	16.3±0.1	10.8±1.2
Fumarate	0.8±0.0	0.8±0.0
Succinate	4.9±0.8	8.1±1.0
Citrate	2.0±0.1	0.9±0.0
Isocitrate	N.D.	N.D.
2-oxoglutarate	1.2±0.1	0.8±0.1

[a] Intracellular metabolites were extracted as described in the Materials and Methods. [b] N.D., not detected. The data from two independent experiments are shown as means ± standard errors (SEs).

DISCUSSION

The present study identified *C. glutamicum NCgl2130* as a novel gene encoding the succinate exporter SucE1, and I designated this gene *sucE1*. It was shown that SucE1 plays a significant role in succinate production under microaerobic and, particularly, anaerobic conditions. Furthermore, SucE1 was also predicted to belong to the AAE family transporters based on the results of homology searches and hydropathy analyses.

Kinetics of SucE1. In the counterflow assay of SucE1 that was reconstituted into liposomes, the initial uptake rate of succinate was 2.4 nmol, min^{-1}, mg $protein^{-1}$ (Fig. 5). The amino-acid sequence of SucE1 suggested that it belongs to the AAE family of transporters. The aspartate:alanine antiporter AspT from the halophilic Gram-positive lactic-acid bacterium *Tetragenococcus halophilus* has been biochemically characterized previously as a prototype of AAE family members (19,20). The maximum rate of reaction (V_{max}) of AspT for aspartate counterflow was ~1,000 nmol, min^{-1}, mg $protein^{-1}$ (19), which was 400-fold higher than the initial transport rate of succinate by SucE1 (Fig. 5). One possible reason for the difference in the initial rates of SucE1 and AspT could be variation of their Michaelis constants (K_m). In general, the K_m values of exporters for their substrates are higher than those of importers, as described below. The kinetics of bacterial exporters have been analyzed mostly in whole cells: for *C. glutamicum* LysE, the K_m was 20 mM for L-lysine (21,22); for *C. glutamicum* BrnF, the K_m was 21 mM for L-isoleusine (23); and for *C. glutamicum* BrnE, the K_m was 10 mM for L-methionine (24). The respective K_m values of *Staphylococcus aureus* SdcS and *E. coli* DctA, both of which are known succinate importers, were only 6.6 μM (25) and 30

μM (26) for succinate in intact cells.

In the present counterflow experiment for SucE1 (Fig. 5), the succinate concentrations inside and outside the proteoliposome were 100 mM and 100 μM, respectively. Taking into account the general K_m values of exporters, as described above, the concentration of succinate outside the proteoliposomes was expected to be much lower than the probable K_m value of SucE1 for succinate. For the reconstituted SucE1 in proteoliposomes, succinate transport from outside to inside, catalyzed by the enzyme, might be a rate-limiting step due to the external succinate concentration being lower than the expected K_m value of SucE1 for succinate, resulting in the lower initial transport rate. Actually, in the counterflow experiment, increase of succinate concentrate outside the proteoliposome from 100 μM to 1mM led to increase in the initial uptake rate of succinate (data not shown).

Technically, the problem in the counterflow experiment under the high external succinate concentration range over the predicted high *K*m values is the low specific radio activity that leads to a low signal/noise ratio. External specific radio activities for a good signal/noise ratio have not yet been achieved by using commercially available radio active succinate at present. The author hopes to overcome this problem in the near future.

Analyses of SucE1 homologs. Although Saier and colleagues (13) reported that three corynebacterial species had two putative AAE family transporters (NCgl2130 [SucE1] and NCgl0565), my dendrogram suggested that they belonged to different clusters (Fig. 6). SucE1 homologs have also been found in succinate producers such as *E. coli* (14,15), *A. succinogenes* 130Z (16), and *M. succiniciproducens* (17) (Fig. 6). The dendrogram suggested that, with the exception of YbjL from *E. coli*, these homologs

clustered loosely together, not with AspT but rather with SucE1 (Fig. 6). Furthermore, they also had the TMS and TrkA_C domains unique to AAE family transporters.

The TrkA_C domain has been found in the bacterial potassium transporter subunit TrkA, and has been suggested to regulate transporter activity by binding nicotinamide adenine dinucleotide (NAD+) or reduced NAD+ (NADH) (27,28). The TrkA_C domain found in SucE1 might bind NAD+ or NADH and regulate SucE1 activity by reflecting the intracellular NADH/NAD+ ratio under fermentative conditions.

Succinate export and sugar metabolism in *C. glutamicum*. Both the succinate production and the sugar-consumption rate were decreased in the *sucE1*-deletion mutant under microaerobic and anaerobic conditions (Fig. 3). As the result of metabolome analysis, the intracellular levels of succinate and the glycolytic metabolites in the EMP upstream of FBP for the *sucE1Δ* strain under microaerobic conditions were higher than those for the parental strain (Table 3), although the extracellular succinate accumulation was decreased in the culture broth of *sucE1Δ* (Fig. 3(A), (B)). These findings suggested that a loss of SucE1 function caused a failure of succinate removal from the cells, leading to the intracellular accumulation of succinate and glycolytic metabolites that consequently inhibited upstream sugar metabolism. The reason why the intracellular levels of the glycolytic metabolites in the EMP upstream of FBP was increased with the deletion of *sucE1* still remains to be solved. Considering the fact that *C. glutamicum* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was inhibited at high cellular NADH/NAD^+ ratios (29,30), and the possibility that TrkA_C domain found in SucE1 might bind NAD+ or NADH and regulate SucE1 activitiy by reflecting the intracellular NADH/NAD+ ratio, a deletion of *sucE1* might cause an increase in the NADH/NAD+ ratio in fermentative succinate production.

REFERENCES

1. **Asai, Y., Inui, M., Vertès, A., Kobayashi, M., Yukawa, H.,:** Cloning and sequence determination of the aspartase-encoding gene from *Brevibacterium flavum* MJ233, Gene, **158**, 87–90 (1995)
2. **Kurusu, Y., Kainuma, M., Inui, M., Satoh, Y., Yukawa, H.,:** Electroporation-transformation system for coryneform bacteria by auxotrophic complementation, Agric. Biol. Chem., **54**, 443–447 (1990)
3. Miller, J.H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
4. **Nakamura, J., Hirano, S., Ito, H., Wach, M.,**: Mutations of the *Corynebacterium glutamicum* NCgl1221 Gene, Encoding a Mechanosensitive Channel Homolog, Induce L-Glutamic Acid Production, Appl. Environ. Microbiol., **73**, 4491–4498 (2007)
5. **Miwa, K., Matsui, K., Terabe, M., Ito, K., Ishida, M., Takagi, H., Nakamori, S., Sano, K.,:** Construction of novel shuttle vectors and a cosmid vector for the glutamic acid-producing bacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*, Gene, **39**, 281–286 (1995)
6. **Chinen, A., Kozlov, Y. I., Hara, Y., Izui, H., Yasueda, H.,:** Innovative Metabolic Pathway Design for Efficient L-Glutamate Production by Suppressing CO_2 Emission, J. Biosci. Bioeng., **103**, 262–269 (2007)
7. **Sambrook, J., Russell, D.W.,**: Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001)
8. **Gutmann, M., Hoischen, C., Krämer, R.,:** Carrier-mediated glutamate secretion

by *Corynebacterium glutamicum* under biotin limitation, Biochim. Biophys. Acta, **1112**, 115–123 (1992)

9. **Ambudkar, S., Maloney, P.C.,:** Bacterial anion exchange. Use of osmolytes during solubilization and reconstitution of phosphate-linked antiport from *Streptcoccus lactis*, J. Biol. Chem., **261**, 10079–10086 (1986)
10. **Okino, S., Inui, M., Yukawa, H.,**: Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation, Appl. Microbiol. Biotechnol., **68**, 475–480 (2005)
11. **Inui, M., Murakami, S., Okino, S., Kawaguchi, H., Vertès, A.A., Yukawa, H.,:** Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions, J. Mol. Microbiol. Biotechnol., **7**, 182–196 (2004)
12. **Engels, V., Linder, S., Wendisch, V. F.,:** The global repressor SugR controls expression of genes of glycolysis and of the L-lactate dehydrogenase LdhAA in *Corynebacterium glutamicum*, J. Bacteriol., **190**, 8033–8044 (2008)
13. **Barabote, R.D., Tamang, D.G., Abeywardena, S.N., Fallah, N.S., Fu, J.Y., Lio, J.K., Mirhosseini, P., Pezeshk, R., Podell, S., Salampessy, M.L., Thever, M.D., Saier, M.H. Jr.,:** Extra domains in secondary transport carriers and channel proteins, Biochim. Biophys. Acta, **1758**, 1557–1579 (2006)
14. **Donnelly, M.I., Millard, C.S., Stols, L.,:** Mutant *E. coli* strain with increased succinic acid production, U.S. patent 5770435 (1998)
15. **Nghiem, N.P., Donnelly, M.I., Millard, C.S., Stols, L.,:** Method for the production of dicarboxylic acids, U.S. patent 5869301 (1999)
16. **Guettler, M.V., Rumler, D., Jain, M.K.,:** *Actinobacillus succinogenes* sp. nov., a

novel succinic-acid-producing strain from the bovine rumen, Int. J. Syst. Evol. Microbiol., **49**, 207–216 (1999)

17. **Lee, P.C., Lee, S.Y., Hong, S.H., Chang, H.N.,:** Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen, Appl. Microbiol. Biotechnol., **58**, 663–668 (2002)
18. **Abe, K., Hayashi, H., Maloney, P.C.,:** Exchange of aspartate and alanine. Mechanism for development of a proton-motive force in bacteria, J. Biol. Chem., **271**, 3079–3084 (1996)
19. **Nanatani, K., Maloney, P.C., Abe, K.,:** Structural and functional importance of transmembrane domain 3 (TM3) in the aspartate:alanine antiporter AspT: topology and function of the residue of TM3 and oligomerization of AspT, J. Bacteriol., **191**, 2122–2132 (2009)
20. **Abe, K., Ohnishi, F., Yagi, K., Nakajima, T., Higuchi, T., Sano, M., Machida, M., Sarker, R.I., Maloney, P.C.,:** Plasmid-encoded asp operon confers a proton motive metabolic cycle catalyzed by an aspartate-alanine exchange reaction, J. Bacteriol., **184**, 2906–2913 (2002)
21. **Bröer, S., Krämer, R.,:** Lysine secretion by *Corynebacterium glutamicum*. 1. Identification of a specific secretion carrier system, Eur. J. Biochem., **202**, 131–135 (1991)
22. **Vrljic, M., Sahm, H., Eggeling, L.,:** A new type of translocator with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*, Mol. Microbiol., **22**, 815–826 (1996)
23. **Zittrich, S., Krämer, R.,:** Quantitative discrimination of carrier-mediated excretion of isoleucine from uptake and diffusion in *Corynebacterium glutamicum*, J.

Bacteriol., **176**, 6892–6899 (1994)

24. **Trötschel, C., Deutenberg, D., Bathe, B., Burkovski, A., Krämer, R.,:** Characterization of Methionine Export in *Corynebacterium glutamicum*, J. Bacteriol., **187**, 3786–3794 (2005)
25. **Hall, J. A., Pajor, A. M.,:** Functional Characterization of a Na^+-Coupled Dicarboxylate Carrier Protein from *Staphylococcus aureus*, J. Bacteriol., **187**, 5189–5194 (2005)
26. **Kay, W.W., Kornberg, H.L.,:** The Uptake of C_4-Dicarboxylic Acids by *Escherichia coli*, Eur. J. Biochem., **18**, 274–281 (1971)
27. **Schlösser, A., Hamann, A., Bossemeyer, D., Schneider, E., Bakker, E.P.,:** NAD+ binding to the *Escherichia coli* K(+)-uptake protein TrkA and sequence similarity between TrkA and domains of a family of dehydrogenases suggest a role for NAD+ in bacterial transport, Mol. Microbiol., **9**, 533–543 (1993)
28. **Anantharaman, V., Koonin, E.V., Aravind, L.,:** Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains, J. Mol. Biol., **307**, 1271-1292 (2001)
29. **Dominguez, H., Rollin, C., Guyonvarch, A., Guerquin-Kern, J., Cocaign-Bousquet, M., Lindley, N.D.,:** Carbon-flux distribution in the central metabolic pathways of *Corynebacterium glutamicum* during growth of fructose, Eur. J. Biochem., **254**, 96–102 (1998)
30. **Omumasaba, C.A., Okai, N., Inui, M., Yukawa, H.,:** *Corynebacterium glutamicum* Glyceraldehyde-3-Phosphate Dehydrogenase Isoforms with Opposite, ATP-Dependent Regulation, J. Mol. Microbiol. Biotechnol., **8**, 91–103 (2004)

Summary

Corynebacterium glutamicum produces succinate from glucose via the reductive tricarboxylic acid cycle under microaerobic and anaerobic conditions. The author identified a *NCgl2130* gene of *C. glutamicum* as a novel succinate exporter that functions in succinate production, and designated *sucE1*. *sucE1* expression levels were higher under microaerobic conditions than aerobic conditions, and overexpression or disruption of *sucE1* respectively increased or decreased succinate productivity during fermentation. Under microaerobic conditions, the *sucE1* disruptant *sucE1Δ* showed 30 % less succinate productivity and a lower sugar-consumption rate than the parental strain. Under anaerobic conditions, succinate production by *sucE1Δ* ceased. The intracellular succinate and fructose-1,6-bisphosphate levels of *sucE1Δ* under microaerobic conditions were respectively 1.7-fold and 1.6-fold higher than those of the parental strain, suggesting that loss of SucE1 function caused a failure of succinate removal from the cells, leading to intracellular accumulation that inhibited upstream sugar metabolism. Homology and transmembrane helix searches identified SucE1 as a membrane protein belonging to the aspartate:alanine exchanger (AAE) family. Partially purified 6x-histidine-tagged SucE1 (SucE1-$[His]_6$) reconstituted in succinate-loaded liposomes clearly demonstrated counterflow and self-exchange activities for succinate. Together, these findings suggest that *sucE1* encodes a novel succinate exporter that is induced under microaerobic conditions, and is important for succinate production under both microaerobic and anaerobic conditions.

CHAPTER II

Identification of *yjjPB* gene encoding succinate transporter in *Escherichia coli* important for succinate production and analysis of the homologs in *Enterobacter aerogenes*

SECTION 1

Identification of *yjjPB* gene encoding succinate transporter in *Escherichia coli* important for succinate production

In this study, I report a genetic screen conducted to identify succinate transporters in *E. coli* and the characterization of YjjPB as a candidate succinate transporter, which participates in succinate production in *E. coli* under both aerobic and anaerobic conditions and possess a completely different structure and conserved domains compared to known bacterial succinate transporters. Furthermore, I found that other known succinate producers, such as *A. succiniciproducens*, *A. succinogenes*, and *M. succiniciproducens*, have YjjPB homologs with high similarity. My findings could be applied to improve industrial succinate production.

Materials and methods

Bacterial strains and culturing. Details of the strains and plasmids used in this study are summarized in Table 1. Plasmids were introduced into *E. coli*, *P. ananatis*, and *C. glutamicum* by electro-transformation (1). *E. coli* was grown in Luria-Bertani (1)

(LB) or LB containing 5% glucose and M9 salts (1) (LBGM9) medium at 37 °C. *P. ananatis* was grown in LBGM9 medium at 34 °C. *C. glutamicum* was grown in CM-Dex medium (2) at 31.5 °C. The following antibiotics were used to select for transformants and maintain plasmids: 25 mg L^{-1} kanamycin in *C. glutamicum* and 100 mg L^{-1} ampicillin, 40 mg L^{-1} kanamycin, 12.5 mg L^{-1} tetracycline, and 25 mg L^{-1} chloramphenicol in *E.coli* and *P. ananatis*. All bacteria were shaken in the test tube or Sakaguchi flask which were capped with silicosen (Shin-Etsu Polymer, Tokyo, Japan) for growth, except for succinate production.

Table 1 Bacterial strains and plasmids used in this study

Strains and Plasmids	Description	Antibiotic resistance[1]	Reference or source
Strains			
FKSP4	*P. ananatis* SC17(0)Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP	Tet	(3)
FKSP37	*P. ananatis* SC17(0)Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP/pSTV28	Tet, Cm	This work
FKSP58	*P. ananatis* SC17(0)Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP/pSTV28-1655_4	Tet, Cm	This work
FKSP68	*P. ananatis* SC17(0)Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP/pSTV28-yjjPB	Tet, Cm	This work
FKSP70	*P. ananatis* SC17(0)Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP/pSTV28-yjjP	Tet, Cm	This work
FKSP97	*P. ananatis* SC17(0)Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP/pSTV28-yjjB	Tet, Cm	This work
AJ110655	*C. glutamicum* MJ233Δ*ldh*	None	(4)
FK948	*C. glutamicum* AJ110655Δ*sucE1*	None	(4)
FK902	*C. glutamicum* AJ110655/pVK9	Km	(4)
FKS3	*C. glutamicum* FK948/pVK9	Km	(4)
FKS168	*C. glutamicum* FK948/pVK9::PmsrA-yjjPB	Km	This work
FKS169	*C. glutamicum* FK948/pVK9::PmsrA-yjjP	Km	This work
FKS170	*C. glutamicum* FK948/pVK9::PmsrA-yjjB	Km	This work

FKSE156	*E. coli* MG1655 Δ*gltA*Δ*sdhA*Δ*aceK*Δ*glcB*Δ*gclA*Δ*maeA*Δ*maeB* Δ*adhE*Δ*ldhA*Δ*poxB*Δ*ackA*Δ*pta*Δ*lld*Δ*ghrA*Δ*acs*Δ*ptsG*Δ*gadA* Δ*gadB*Δ*ghrB, Ptac-ppc, Ptac-sucCD, Ptac-ybjL*	None	This work
FKSE157	*E. coli* FKSE156Δ*yjjPB*	None	This work
FKSE163	*E. coli* FKSE157 /pSTV28	Cm	This work
FKSE172	*E. coli* FKSE157 /pSTV-yjjPB	Cm	This work
Plasmids			
pSTV28	Plasmid vector containing the pACYC184 origin of replication	Cm	This work
pSTV28-1655_4	Succinate resistant plasmid isolated from the *E. coli* MG1655 genome library	Cm	This work
pFKS1	pTSV28 containing the *E.coli yjjPB* genes under control of the native promoter	Cm	This work
pFKS2	pTSV28 containing the *E.coli yjjP* gene under control of the native promoter	Cm	This work
pFKS3	pTSV28 containing the *E.coli yjjB* gene under control of the *tac* promoter	Cm	This work
RSFPP	Plasmid for expression of the *E. coli prpC* and *ppc* genes containing the RSF1010 origin of replication	Tet	(3)
pRSFRedTER	Broad-host-range λ Red expressing plasmid	Cm	(5,6)
pMW-$attL_{\lambda}$-Km^{R}-$attR_{\lambda}$	Cassette for gene disruption containing the kanamycin resistance gene	Km	(5,7)
pMWts-λInt/Xis	λ Int/Xis expressing plasmid	Ap	(7,8)
pRSF-*Para*-IX	Broad-host-range λ Int/Xis expressing plasmid	Cm	(6)
pVK9	An *E. coli* and *C. glutamicum* shuttle vector	Km	(9)
pFKS4	pVK9 containing the *E.coli yjjP* gene under control of the *msrA* promoter	Km	This work
pFKS5	pVK9 containing the *E.coli yjjB* gene under control of the *msrA* promoter	Km	This work
pFKS6	pVK9 containing the *E.coli yjjPB* genes under control of the *msrA* promoter	Km	This work

[1]Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol; Tet, tetracycline

Plasmid construction. To construct plasmids pFKS6, pFKS4, and pFKS5, the *msrA* promoter from the chromosomal DNA of *C. glutamicum* AJ13869 (10) was amplified with primers PmsrA-F/PmsrA-R. PrimeSTAR Max DNA Polymerase (Takara Bio, Shiga, Japan) was used in PCR experiments. The chromosomal DNA of all bacteria was extracted using the PurElute™ Bacterial Genomic Kit (EdgeBio, Gaithersburg, State of Maryland, USA). The *yjjPB*, *yjjP*, or *yjjB* genes were amplified from the chromosomal DNA of *E. coli* MG1655 with primers CyjjPB-F/CyjjPB-R, CyjjPB-F/CyjjP-R, and CyjjB-F/CyjjPB-R, respectively. Next, the *msrA* promoter and each gene fragment were inserted into *Bam*HI- and *Pst*I- digested plasmid pVK9 (9) 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 (9) into plasmid pHSG299 (Takara Bio).

To construct plasmids pFKS1 and pFKS2 in which the genes are expressed under their native promoters, the *yjjPB* and *yjjP* genes were amplified using chromosomal DNA from *E. coli* MG1655 with primers yjjP-F/yjjPB-R and yjjP-F/yjjP-R, respectively. The amplified DNA fragments were then inserted into *BamH*I- and *Pst*I-digested plasmid pSTV28 using the In-Fusion HD Cloning Kit. To construct plasmid pFKS3, the *tac* promoter was amplified using the pTWV-P_{tac}-T_{trp} vector (11) as the DNA template with primers Ptac-F/Ptac-R. The *yjjB* gene was amplified using chromosomal DNA from *E. coli* MG1655 with primers yjjB-F/yjjPB-R. The amplified DNA fragment was inserted into the *BamH*I and *Pst*I sites of plasmid pSTV28 using the In-Fusion HD Cloning Kit.

Plasmid RSFPP was constructed from plasmid RSFPPG (25), which carries the *E. coli prpC*, *ppc*, and *gdhA* genes. RSFPPG was digested with *Nsp*V and then self-ligated.

The resulting plasmid, from which only the *gdhA* gene was deleted, was designated as RSFPP.

Disruption and amplification of genes in *E. coli*. P1 transduction was used with the Keio collection (12) to disrupt the *gltA*, *sdhA*, *aceK*, *glcB*, *gclA*, *maeA*, *maeB*, *adhE*, *ldhA*, *poxB*, *ackA-pta*, *lld*, *ghrA*, *acs*, *ptsG*, *gadA*, *gadB*, *ghrB*, and *yjjP*. Antibiotic resistant strains were selected as correct transformants. To remove the antibiotic marker, the λ Int/Xis-driven system was used with the pMWts-λInt/Xis-helper plasmid, which carries the genes encoding the λ phage integrase (Int) and the excisionase (Xis) and enables temperature-sensitive replication (7,13).

To overexpress the *ppc* gene, the original promoter was changed to the *tac* promoter with the λ Red system using pKD46 as the helper plasmid (5). A removable kanamycin resistance gene flanked by $attL_{\lambda}$ and $attR_{\lambda}$ was amplified with primers Ptac-ppc-F/Ptac-ppc-R, which contain 60-nt sequences homologous to the target region of the chromosome, using plasmid pMW-$attL_{\lambda}$-Km^R-$attR_{\lambda}$[29] as the DNA template. The resulting fragment was transformed into a strain containing plasmid pKD46 and transformants were selected on LB plates containing 100 mg L^{-1} ampicillin and 40 mg L^{-1} kanamycin at 30 °C. Antibiotic resistant strains were designated as P_{tac}-*ppc*::Km. The antibiotic marker was removed using the λ Int/Xis-driven system with the pMWts-λInt/Xis-helper plasmid.

The *sucCD* and *ybjL* genes were overexpressed similarly by changing the native promoters to the *tac* promoter using the λ Red system. Fragments containing a removable kanamycin resistance gene flanked by $attL_{\lambda}$ and $attR_{\lambda}$ were amplified with primer pairs Ptac-sucCD-F/Ptac-sucCD-R and Ptac-ybjL-F/Ptac-ybjL-R, respectively.

Gene disruption in *P. ananatis*. To disrupt the *sdhA* gene, the λ Red gene knockout system was used with the Red-recombining helper plasmid pRSFRedTER (5, 6). A removable kanamycin resistance gene flanked by $attL_{\lambda}$ and $attR_{\lambda}$ was amplified using primers Δ*sdhA*-*attL*/Δ*sdhA*-*attR*, which contain 50-nt sequences homologous to the target region at the 5′-end of the chromosome, with plasmid pMW-$attL_{\lambda}$-Km^R-$attR_{\lambda}$ as the DNA template (5,7). Antibiotic resistant transformants were designated as strain SC17(0) Δ*sdhA*::Km. To remove pRSFRedTER, the strains were streaked out on LB plates containing 10% sucrose and 1 mM isopropyl β-D-1-thiogalactopyranoside to obtain single colonies. Removal of pRSFRedTER was confirmed using LB plates containing 40 mg L^{-1} chloramphenicol. Strains unable to grow under these conditions were designated as strain pRSFRedTER-free SC17(0) Δ*sdhA*::Km. All primer sequences are listed in Table 2. Next, the λ Int/Xis-driven system was used with the pRSF-P_{ara}-IX-helper plasmid (6) to remove the kanamycin marker. pRSF-P_{ara}-IX was introduced into the marker strain via electro-transformation. Transformants harboring pRSF-P_{ara}-IX were selected on LB plates containing 25 mg L^{-1} chloramphenicol. The resulting transformants were then streaked onto LB plates containing 25 mg L^{-1} chloramphenicol and 1% L-arabinose; clones that formed single colonies were identified and elimination of the kanamycin resistance gene was confirmed by growth on LB medium containing 40 mg L^{-1} kanamycin. Strains unable to grow on kanamycin plates were utilized as marker-free strains and designated as SC17(0)Δ*sdhA* strains. pRSF-P_{ara}-IX was removed from the marker-free strain as described for pRSFRedTER.

Table 2 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*	GCATATGTATGACACCGTCAAAGGTTCCGACTACATCGGTGACCAGGACGTG AAGCCTGCTTTTTTATACTAAGTTGGCA
Δ*sdhA-attR*	TCCAGCTCAAGGCACTCAATACGCTGTGTATTGAAGTCAGGTGAGCGGTCCG CTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ppc-F	TCAGGTGTGTTTAAAGCTGTTCTGCTGGGCAATACCCTGCAGTTTCGGGTCG CTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ppc-R	GTGAAGGATACAGGGCTATCAAACGATAAGATGGGGTGTCTGGGGTAATTGA AGCCTGCTTTTTTATACTAAGTTGGCA
Ptac-sucCD-F	TTACTGCTGCTGTGCAGACTGAATCGCAGTCAGCGCGATGGTGTAGACGACG CTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-sucCD-R	CCGCCATATGAACGGCGGGTTAAAATATTTACAACTTAGCAATCAACCATGAA GCCTGCTTTTTTATACTAAGTTGGCA
Ptac-ybjL-F	TTACCTTAGCCAGTTTGTTTTCGCCAGTTCGATCACTTCATCACCGCGTCCGC TCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ybjL-R	ACACTAGTAAAATATATTGTTACTTTACTATCGTTTAGGTGCGCTGAATTGAAG CCTGCTTTTTTATACTAAGTTGGCA

Screening for genes encoding succinate transporters in *E. coli*. *P. ananatis* SC17(0)Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP (3) was used as the host strain for the screen. This strain was transformed with the *E. coli* MG1655 genome library and then plated on minimal media (pH 4.7) containing 200 mM L-Glu and 1 mM succinate. Plasmids were isolated from strains able to grow on the screening plates and then used to re-transform SC17(0)Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP. The isolated plasmids were used for sub-cloning the succinate transporter-encoding gene.

Succinate fermentation under aerobic conditions. Pre-culturing was performed on LBGM9 plates at 34 °C for 16 h. The cells were then inoculated into test tubes containing 5 mL succinate fermentation medium [30 g L^{-1} sucrose, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 g L^{-1} yeast extract, 5 g L^{-1} $(NH_4)_2SO_4 \cdot 7H_2O$, 2 g L^{-1} KH_2PO_4, 10 mg L^{-1} $MnSO_4 \cdot 5H_2O$, and 10 mg L^{-1} $FeSO_4 \cdot 7H_2O$] containing 20 g L^{-1} precipitated $CaCO_3$ sterilized by dry heat at 180 °C for 3 h (Japanese Pharmacopoeia, Tokyo, Japan). Following inoculation, succinate fermentation was performed at 34 °C and a rotation speed of 120 rpm for 20 h.

Succinate fermentation under anaerobic conditions. To examine succinate production in *E. coli* or *C. glutamicum* under anaerobic conditions, we employed a convenient evaluation system utilizing 1.5-mL microcentrifuge tubes (4,6). *E. coli* cells were pre-cultured on LBGM9 plates at 37 °C for 18 h. The pre-cultured cells were washed twice with cold *E. coli* anaerobic medium without $CaCO_3$ and then inoculated into 1.5-mL microcentrifuge tubes containing 1.3 mL of *E. coli* anaerobic medium [15 g L^{-1} glucose, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 g L^{-1} Bacto yeast extract, 20 g L^{-1} $(NH_4)_2SO_4 \cdot 7H_2O$, 2 g L^{-1} KH_2PO_4, 20 mg L^{-1} $MnSO_4 \cdot 5H_2O$, 20 mg L^{-1} $FeSO_4 \cdot 7H_2O$, and 15 g L^{-1} MOPS (pH 7.0 with KOH)] containing 50 g L^{-1} precipitated $CaCO_3$

sterilized by dry heat at 180 °C for 3 h (Japanese Pharmacopoeia, Tokyo, Japan). The tubes were tightly capped and succinate fermentation was performed using an Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany) at 30 °C with a rotation speed of 1,400 rpm. Initial biomass was adjusted to an optical density (OD_{600nm}) of 10-15**.** *C. glutamicum* 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} thiamin·HCl (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 31.5 °C for 48 h under anaerobic conditions.

Metabolite analysis. Organic acids that accumulated in the medium were analyzed by high-performance liquid chromatography (HPLC) on a CDD-10AD system (Shimadzu Co. Ltd., Kyoto, Japan) using a suitable dilution as described in the atuhor's previous report.[12)] Glucose concentrations were analyzed using an AS-310 Biotech Analyzer (Sakura SI Co. Ltd., Tokyo, Japan). OD was measured at 620 nm with a U-2001 spectrometer (Hitachi Co. Ltd, Tokyo, Japan). Broth containing $CaCO_3$ was diluted with 0.1 N HCl prior to measuring the OD_{620}.

Construction of the *E. coli* genome library. Chromosomal DNA of *E.coli* MG1655 was extracted using the PurElute™ Bacterial Genomic Kit (EdgeBio). The pSTV28 vector was used for the genome library. The genome library was constructed using the outsourcing service of TAKARA BIO (Shiga, Japan). The library contains

about 37,000 clones μl^{-1}, with inserts in size more than 4 kb, representing theoretically a 32-fold coverage of the *E.coli* MG1655 genome.

Hydropathy and sequence-similarity analyses of YjjPB. The YjjPB hydropathy analyses were conducted using SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/). Sequence similarity analyses of YjjPB were conducted using the Kyoto Encyclopedia of Genes and Genomes Sequence Similarity Database (KEGG SSDB; http://www.genome.jp/kegg/ssdb/). Sequence similarities between YjjPB and homologs from various other bacteria were analyzed using GENETYX software ver.10. To identify conserved domain sequences, each sequence was analyzed using the Pfam protein families database (Pfam; http://pfam.xfam.org/).

Results

Screening for a succinate transporter in *E. coli*. In order to identify genes encoding a succinate transporter in *E. coli*, I constructed a screening system using *P. ananatis* FKSP4 (3) as the host strain. The strain was constructed from the aerobic condition succinate producer strain SC17(0)Δ*sdhA*/RSFPP by disrupting the *yeeA* and *ynfM* genes, which I hypothesize encode putative dicarboxylate transporters (in preparation). Plasmid RSFPP carries the *prpC* and *ppc* genes in order to increase TCA cycle flux. FKSP4 shows sensitivity in the presence of 1 mM succinate under acidic conditions (pH 4.7). At pH4.7, a certain percentage of succinic acid exists in the free form with two protonated carboxyl groups; the protonated form can permeate the plasma membrane without any transporter. Permeated succinic acid can intracellularly liberate protons and be converted into either the monoanionic form of succinate

(succinate^{1-}) or dianionic form of succinate (succinate^{2-}) because the intracellular pH is generally neutral. Furthermore, strain SC17(0)Δ*sdhA*/RSFPP is unable to degrade succinate^{1-} and succinate^{2-} because of the *sdhA* deletion, but can produce succinate from glucose present in the plate. I hypothesized that the sensitivity of cells to succinate in the plates would depend on the intracellular succinate concentration and that strains would be able to grow on a plate containing 1 mM succinate under acidic condition if succinate efflux activity increased.

Using this system with the *E. coli* MG1655 genome library, obtained several transformants able to grow on the screening plate were obtained. Of these, pSTV28-1655_4 was isolated from two transformants that grew faster on this plate. In addition, the re-transformant of SC17(0)Δ*sdhA*/RSFPP by extracted pSTV28-1655_4 indicated the complementation of its growth. Sequence analysis of pSTV28-1655_4 revealed that the putative membrane protein genes, *yjjP* and *yjjB,* and the regulator gene, *yjjQ* were present in the plasmid.

Next, I investigated whether introduction of pSTV28-1655_4 increases succinate production levels under aerobic conditions using FKSP4 as the background strain. Succinate production of strain FKSP37 (control – FKSP4/pSTV28) was 0.8 g L^{-1}, while that of strain FKSP58 (FKSP4/pSTV28-1655_4) was greater than 7 g L^{-1} (Fig. 1A). These results indicate that pSTV28-1655_4 contains factors that increase succinate production under aerobic conditions.

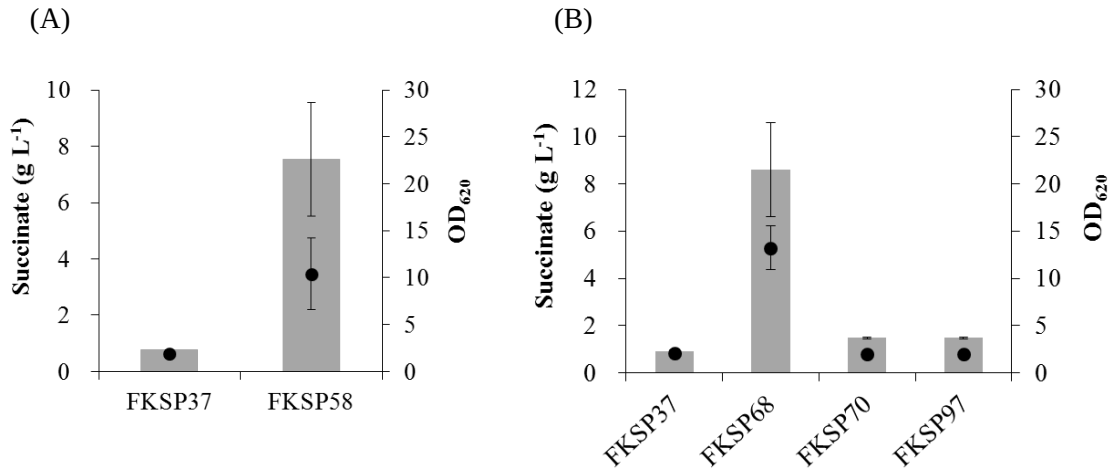

Fig.1. Effect of introducing pSTV28-1655_4 and expression of the *yjjP*, *yjjB*, or *yjjPB* genes on succinate production under aerobic conditions.

P. ananatis strain FKSP4 was used as the host. Succinate production was measured as described in the Materials and Methods. OD_{620} (●), extracellular succinate concentration (gray columns). (A) Effect of introducing pSTV28-1655_4 isolated from a succinate-resistant strain. *P. ananatis* FKSP37 (control – FKSP4/pSTV28) and FKSP58 (FKSP4/pSTV28-1655_4) were used. (B) Effect of *yjjP*, *yjjB*, or *yjjPB* expression. *P. ananatis* FKSP37 (control – FKSP4/pSTV28), FKSP68 (FKSP4/pSTV28-yjjPB), FKSP70 (FKSP4/pSTV28-yjjP) and FKSP97 (FKSP4/pSTV28-yjjB) were used. The data from three independent experiments are shown as means, SEs.

Effect of *yjjP*, *yjjB*, or *yjjPB* expression on succinate production under aerobic conditions. The putative amino acid sequences of the proteins encoded by the *yjjP*, *yjjB* and *yjjQ* genes found on pSTV28-1655_4 were subjected to bioinformatic analysis.

Hydropathy analysis using the SOSUI prediction program (http://harrier.nagahama-i-bio.ac.jp/sosui/) indicated that YjjP and YjjB, which form the predicted membrane proteins, consist of 256 and 157 amino acids, respectively (Fig. 2). In addition, hydropathy analysis using the SOSUI prediction program indicates that YjjP and YjjB possess 5 and 4 transmembrane domains, respectively (Fig. 2). YjjQ is predicted to be a regulatory protein.

In order to confirm whether these putative membrane proteins are responsible for the increased succinate production, *yjjP* and/or *yjjB* were subcloned into pSTV28 and the resulting plasmids were transformed into strain FKSP4. Fig. 1B shows the results of succinate fermentation under aerobic conditions. Succinate production was significantly increased by expression of *yjjPB* under its native promoter (from 0.93 g L^{-1} to 8.6 g L^{-1}), while no significant increase was apparent when *yjjP* or *yjjB* were expressed independently (and not as an operon) under the control of the native or *tac* promoter, respectively (Fig. 1B). In addition, succinate production was not affected by the expression of *yjjQ* (data not shown). These results indicate that the factors present on pSTV28-1655_4 responsible for increasing succinate production are the *yjjPB* genes and that these genes contribute to succinate production under aerobic conditions.

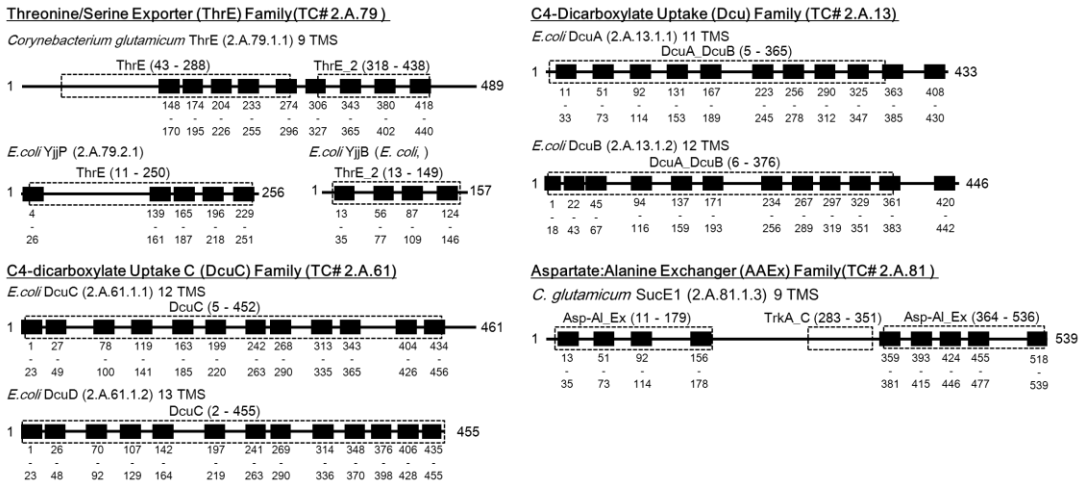

Fig. 2. The transmembrane helices and conserved domains of YjjPB, ThrE, and other known bacterial succinate transporters. The SOSUi transmembrane helix prediction programs (14) and the Pfam database (15) were used for model construction. The putative transmembrane helices are indicated by black boxes and the conserved domains by stippled boxes.

Complementation of *sucE1* deletion by *yjjP, yjjB,* and *yjjPB* in *C. glutamicum*. Previously, I identified a novel succinate transporter, SucE1, in *C. glutamicum* (4). Furthermore, I demonstrated that the *sucE1*-deletion mutant could not produce succinate because it lacked a succinate export carrier and that complementation of the *sucE1* gene by plasmid transformation restored succinate production under these conditions (4). Therefore, I next examined whether succinate production could be complemented by introducing the *yjjPB*, *yjjP*, or *yjjB* genes into *C. glutamicum* strain FK948, which cannot produce succinate because of a disruption of succinate transporter, SucE1. *yjjP*, *yjjB*, or *yjjPB* were cloned into pVK9 and expressed under the

control of the *C. glutamicum msrA* (methionine sulfoxide reductase) promoter. These plasmids were then introduced into strain FK948. Fig. 3 shows the results for succinate fermentation conducted in 1.5-mL microcentrifuge tubes under anaerobic conditions. Strain FK902 (control – AJ110655/pVK9) produced 50 g L^{-1} succinate, while FKS3 (AJ110655Δ*sucE1*/pVK9) produced only 1 g L^{-1} succinate. Strain FKS169 (AJ110655Δ*sucE1*/pVK9::PmsrA-yjjP) and FKS170 (AJ110655Δ*sucE1*/pVK9::PmsrA-yjjB) produced 1.2 and 1.6 g L^{-1} succinate, respectively. However, succinate production was restored in strain FKS168 (AJ110655Δ*sucE1*/pVK9::PmsrA-yjjPB) to 14 g L^{-1} . These results demonstrate that expression of YjjPB partly complemented SucE1 function and that both proteins are required for complementation.

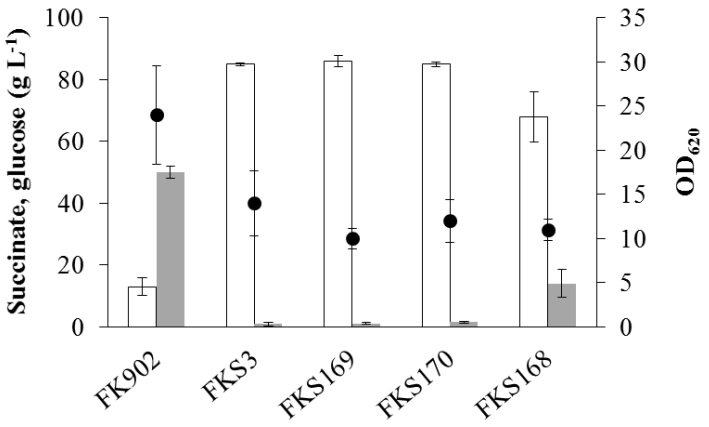

Fig. 3. Complementation of the *sucE1* deletion with *yjjP*, *yjjB*, and *yjjPB* in *C. glutamicum*

E. coli yjjP and *yjjB* were introduced into *C. glutamicum* FK948, a strain in which the limiting step for succinate production is succinate export. *C. glutamicum* FK902 (control – AJ110655/pVK9), FKS3 (AJ110655Δ*sucE1*/pVK9), FKS169 (AJ110655Δ*sucE1*/pVK9::PmsrA-yjjP), FKS170 (AJ110655Δ*sucE1*/pVK9::PmsrA-yjjB) and FKS168 (AJ110655Δ*sucE1*/pVK9::PmsrA-yjjPB) were used. Fermentation was performed in 1.5-mL microcentrifuge tubes as described in the Materials and Methods. OD_{620} (●), residual glucose (unfilled columns), succinate concentration (gray columns). The data from four independent experiments are shown as means, SEs.

Effect of *yjjPB* deletion on succinate production in *E. coli* under anaerobic conditions. Next, I examined the effect of deleting the *yjjPB* genes on succinate production in *E. coli* under anaerobic conditions. *E. coli* strain FKSE156 was used as a model-succinate producer. In this strain, the *adhE*, *ldhA*, and *ackA-ptaA*/*poxB* genes were disrupted in order to decrease ethanol, lactate, and acetate production, respectively,

and *ppc*, which is known to be important for succinate production (16, 17), was overexpressed. The fermentation results show that succinate accumulation (extracellular concentration) was 6.8 g L^{-1} for strain FKSE156 (control) and 2.0 g L^{-1} for strain FKSE157 (FKSE156Δ*yjjPB*) after 20 h (Fig. 4A). Succinate production was decreased by 70% following deletion of the *yjjPB* genes. The residual glucose concentration was 1.6 g L^{-1} in strain FKSE156 and 7.6 g L^{-1} in strain FKSE157. Glucose consumption was also decreased by 40% following deletion of *yjjPB*. Furthermore, succinate accumulation and residual glucose concentration were 6.6 g/L and 2.8 g/L for strain FKSE172 (*yjjPB* complementation), comparable to the values of strain FKSE156 (Fig. 4B), suggesting that pFKS1 expressed *yjjPB* at a much higher level than the endogenous *yjjPB* genes present on the chromosome. These results demonstrate that YjjPB mainly contribute to enhancing succinate production under anaerobic conditions.

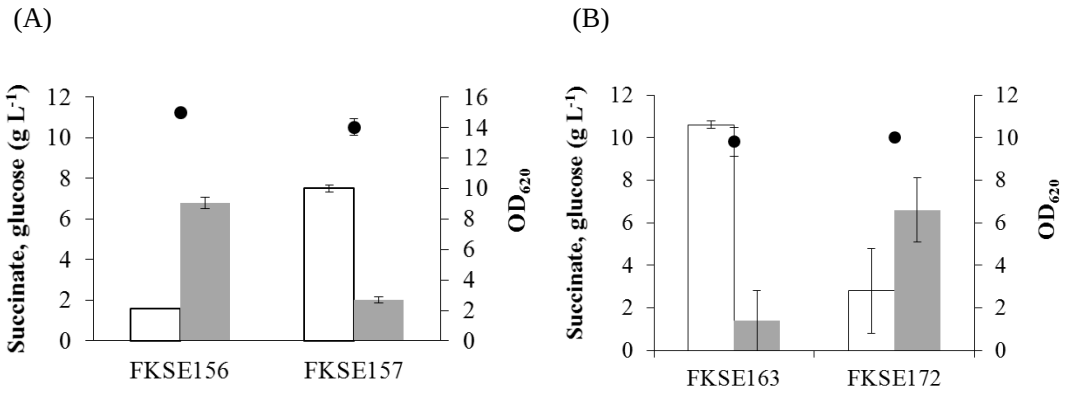

Fig.4. Effect of *yjjPB* deletion on succinate production in *E. coli* under anaerobic conditions. Strain FKSE156 was used as the host. Succinate production was performed as described in the Materials and Methods; culturing time was 20 h. OD_{620} (●), residual glucose (unfilled columns), succinate concentration (gray columns). (A) Effect of *yjjPB* deletion on succinate production. *E. coli* FKSE156 (control) and FKSE157 (FKSE156Δ*yjjPB*) were used. (B) Effect of *yjjPB* expression on succinate production in a Δ*yjjPB* strain. *E. coli* FKSE163 (control – FKSE156Δ*yjjPB*/pSTV28) and FKSE172 (FKSE156Δ*yjjPB*/pSTV-yjjPB) were used.The data from three independent experiments are shown as means, SEs.

Hydropathy and sequence-similarity analyses of YjjPB. *C. glutamicum* (4), *Enterobacter aerogenes* (6), *A. succinogenes* 130Z (18), and *M. succiniciproducens* (19, 20) are known to produce succinate under anaerobic conditions. Therefore, I examined whether these bacteria possess YjjPB homologs. A BLAST search using the amino-acid sequences of YjjP and YjjB revealed that *E. aerogenes*, *A. succinogenes*, and *M. succiniciproducens* (but not *C. glutamicum*) have both YjjP and YjjB homologs. The predicted amino-acid sequences of the *E. aerogenes*, *A. succinogenes*, and *M. succiniciproducens* YjjP homologs exhibited 88% (99%), 50% (87%), and 54% (89%)

identity (similarity), respectively, with *E. coli* YjjP. All YjjP homologs possess 4-6 putative transmembrane domains. The predicted amino-acid sequences of the *E. aerogenes, A. succinogenes*, and *M. succiniciproducens* YjjB homologs exhibited 83% (97%), 51% (81%), and 52% (83%) identity (similarity), respectively, with *E. coli* YjjB and all YjjB homologs possess 4 putative transmembrane domains. These findings suggest that YjjPB might also contribute to enhancing succinate production in these gram-negative succinate producers. Furthermore, the *Shigella flexneri* homologs exhibit 100% and 98% sequence identity to *E. coli* YjjP and YjjB, respectively. *Salmonella enterica* also possess YjjPB homologs and exhibits 89% and 98% sequence identity to *E. coli* YjjP and YjjB, respectively, indicating that YjjPB might act as the major succinate transporter that participates in succinate export in many gram-negative bacteria.

Discussion

In this study, I determined that the *yjjPB* genes of *E. coli* contribute to succinate production under aerobic and anaerobic conditions. The author's results indicate that YjjPB participates in succinate export.

Previous studies have determined the transport activity of exporters by measuring intracellular substrate levels (21,22), measuring transport activity using proteoliposomes (4), or inside-out vesicles (23,24). However, these methods are complex, time consuming, and relatively expensive. Here, I developed a complementation 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 are disrupted. The complementation assay indicated that only the combination of YjjP and YjjB restored succinate production (Fig. 3). Furthermore, succinate production under anaerobic conditions in a succinate producing *E. coli* strain was decreased 70% by deleting the *yjjPB* genes (Fig. 4). Furthermore, under aerobic conditions, expression of the *yjjPB* genes in succinate producing *P. ananatis* strain FKSP4 resulted in an increase in succinate production from 0.93 g L^{-1} to 8.6 g L^{-1} (Fig. 1B). Taken together, these results suggest that YjjPB has succinate-export activity.

A conserved domain search using the Pfam database (15) showed that YjjP has a ThrE domain and YjjB has a ThrE_2 domain in their respective peptide sequences (Fig. 2). These domains are conserved in the Threonine/Serine Exporter (ThrE) Family (TC# 2.A.79, Transporter Classification Database (40)). These results indicate that YjjPB constitute a split type ThrE family transporter. In addition, I predicted the transmembrane helices and conserved domains of other known bacterial succinate

transporters (DcuA-D and SucE1; Fig. 2). Furthermore, the predicted amino-acid sequences of the *E. coli* DcuA, DcuB, DcuC, DcuD, and *C. glutamicum* SucE1 exhibited 20% (20%), 14% (20%), 17% (22%), 18% (23%), and 18% (17%) identity, respectively, with *E. coli* YjjP (YjjB). These results suggest that YjjPB possess a completely different structure and conserved domains compared to these known bacterial succinate transporters and that *E. coli* and *C. glutamicum* use different succinate-export systems.

The ThrE Family transporter was originally identified as a PMF-dependent threonine/serine exporter from *C. glutamicum* (26). The ThrE family is broadly conserved in bacteria, archaea, and eukaryotes. The ThrE family transporters of fungi, archaea, and high GC gram-positive bacteria are comprised of single poly-peptide chain, whereas the ThrE transporters of gram-negative bacteria and low GC gram-positive bacteria have split their poly-peptide chains into two fragments (27). In this study, I demonstrate the succinate export function of *E. coli yjjPB*. To the best of the author's knowledge, this is the first report describing the function of a split ThrE transporter. Further studies will be required to elucidate the relationship between the multiple complex structures and the varied substrate specificity of ThrE family transporters.

REFERENCES

1. **Sambrook J. and Russell DW.:** Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor,N. Y. (2001)
2. **Nakamura J, Hirano S, Ito H, Wach M.,:** Mutations of the *Corynebacterium glutamicum* NCgl1221 gene, encoding a mechanosensitive channel homolog, induce L-glutamic acid production. Appl Environ Microbiol., **73**, 4491–4498 (2007)
3. **Hara Y, Fukui K, Chinen A, Suguru Y.:** Method for manufacturing useful substance. International patent WO2015005406A1 (2015)
4. **Fukui K, Koseki C, Yamamoto Y, Nakamura J, Sasahara A, Yuji R, Hashiguchi K, Usuda Y, Matsui K, Kojima H, Abe K.,:** Identification of succinate exporter in *Corynebacterium glutamicum* and its physiological roles under anaerobic conditions. J Biotechnol., **154**, 25–34 (2011)
5. **Katashkina JI, Hara Y, Golubeva LI, Andreeva IG, Kuvaeva TM, Mashko SV.:** Use of the lambda Red-recombineering method for genetic engineering of *Pantoea ananatis*. BMC Mol Biol.,**10**, 34 (2009)
6. **Tajima Y, Yamamoto Y, Fukui K, Nishio Y, Hashiguchi K, Usuda Y, Sode K.,:** Effects of eliminating pyruvate node pathways, and co-expression of heterogeneous carboxylation enzymes, on succinate production by *Enterobacter aerogenes.* Appl Environ Microbiol., **81**, 929–937 (2015)
7. **Minaeva NI, Gak ER, Zimenkov DV, Skorokhodova AY, Biryukova IV, Mashko SV.,:** Dual-In/Out strategy for genes integration into bacterial chromosome: a novel approach to step-by-step construction of plasmid-less marker-less recombinant *E. coli* strains with predesigned genome structure. BMC

Biotechnol., **8**, 63 (2008)

8. **Datsenko KA, Wanner BL.,:** One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA., **7**, 6640–6645 (2000)
9. **Miwa K, Matsui, K, Terabe M, Ito K, Ishida, M, Takagi H, Nakamori S, Sano, K.,:** Construction of novel shuttle vectors and a cosmid vector for the glutamic acid-producing bacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*. Gene., **39**, 281–286 (1985)
10. **Chinen A, Kozlov YI, Hara Y, Izui H, Yasueda H.,:** Innovative metabolic pathway design for efficient L-glutamate production by suppressing CO_2 emission. J Biosci Bioeng., **103**, 262–269 (2007)
11. **Nishio Y, Ogishima S, Ichikawa M, Yamada Y, Usuda Y, Masuda T and Tanaka H.,:** Analysis of L-glutamic acid fermentation by using a dynamic metabolic simulation model of *Escherichia coli*. BMC Sys Biol., **7**, 92–103 (2013)
12. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.,:** Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol., **2**, 2006.0008 (2006)
13. **Datsenko KA, Wanner BL.,:** One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci USA., **97**, 6640–6645 (2000)
14. **Mitaku S, Hirokawa T, Tsuji T.,:** Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. Bioinformatics., **18**, 608–616 (2002)

15. **Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, Salazar GA, Tate J, Bateman A.,:** The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res., **44**, D279–285 (2016)
16. **Millard CS, Chao YP, Liao JC, Donnelly MI.,:** Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in *Escherichia coli*. Appl Environ Microbiol., **62**, 1808–1810 (1996)
17. **Lin H, San KY, Bennett GN.,:** Effect of Sorghum vulgare phosphoenolpyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*. Appl Microbiol Biotechnol., **67**, 515–523 (2005)
18. **Guettler MV, Rumler D, Jain MK.,:** *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen. Int J Syst Evol Microbiol., **49**, 207–216 (1999)
19. **Lee PC, Lee SY, Hong SH, Chang HN.,:** Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl Microbiol Biotechnol., **58**, 663–668 (2005)
20. **Oh IJ, Kim DH, Oh EK, Lee SY, Lee J.,:** Optimization and scale-up of succinic acid production by *Mannheimia succiniciproducens* LPK7, J Microbiol Biotechnol., **19**, 167–171 (2009)
21. **Trötschel C, Deutenberg D, Bathe B, Burkovski A, Krämer R.,:** Characterization of methionine export in *Corynebacterium glutamicum*. J Bacteriol., **187**, 3786–3794 (2005)
22. **Simic P, Sahm H, Eggeling L.,**: L-Threonine export: use of peptide to identify a

new translocator from *Corynebacterium glutamicum*. J Bacteriol., **183**, 5317–5324 (2001)

23. **Bebawy M, Morris MB, Roufogalis BD.,:** A continuous fluorescence assay for the study of P-glycoprotein-mediated drug efflux using inside-out membrane vesicles. Anal Biochem., **268**, 270–277 (1999)
24. **Soemphol W, Tatsuno M, Okada T, Matsutani M, Kataoka N, Yakushi T, Matsushita K.,:** A novel $Na^+(K^+)/H^+$ antiporter plays an important role in the growth of *Acetobacter tropicalis* SKU1100 at high temperatures via regulation of cation and pH homeostasis. J Bacteriol., **211**, 46–55 (2015)
25. **Saier MH, Reddy VS, Tsu BV, Ahmed MS, Li C, Moreno-Hagelsieb G.,:** The Transporter Classification Database (TCDB): recent advances. Nucleic Acids Res., **44**, D372–379 (2016)
26. **Kruse D, Krämer R, Eggeling L, Rieping M, Pfefferle W, Tchieu JH, Chung YJ, Saier MH, Burkovski A.,:** Influence of threonine exporters on threonine production in *Escherichia coli*. Appl Microbiol Biotechnol., **59**, 205–210 (2002)
27. **Yen MR, Tseng YH, Simic P, Sahm H, Eggeling L, Saier MH.,:** The ubiquitous ThrE family of putative transmembrane amino acid efflux transporters. Res Microbiol., **153**, 19–25 (2002)

Summary

Under anaerobic conditions, *Escherichia coli* produces succinate from glucose via the reductive tricarboxylic acid cycle. To date, however, no genes encoding succinate exporters have been established in *E. coli*. Therefore, the author attempted to identify genes encoding succinate exporters by screening an *E. coli* MG1655 genome library. The author identified the *yjjPB* genes as candidates encoding a succinate transporter, which enhanced succinate production in *Pantoea ananatis* under aerobic conditions. A complementation assay conducted in *Corynebacterium glutamicum* strain AJ110655Δ*sucE*1 demonstrated that both YjjP and YjjB are required for the restoration of succinate production. Furthermore, deletion of *yjjPB* decreased succinate production in *E. coli* by 70% under anaerobic conditions. Taken together, these results suggest that YjjPB constitutes a succinate transporter in *E. coli* and that the products of both genes are required for succinate export.

SECTION 2

Analysis of the *yjjPB* homologs in *Enterobacter aerogenes*

No succinate-exporter genes have been identified to date in *E. aerogenes*. Homology analysis indicated that *E. aerogenes* contains YjjP (EaYjjP) and YjjB (EaYjjB) homologs. In the present study, the author evaluated the effect of *EayjjPB* gene expression on succinate production to elucidate whether EaYjjPB contributes to succinate production as a succinate transporter in *E. aerogenes*. Furthermore, the author performed complementation assays of the *C. glutamicum sucE1* deletion mutant with *EayjjP*, *EayjjB*, and *EayjjPB*.

Materials and methods

Bacterial strains and culturing. Details of the strains and plasmids used in this study are summarized in Table 1. Plasmids were introduced into *E. coli*, *C. glutamicum*, and *E. aerogenes* by electro-transformation (1). *E. coli* was grown in Luria-Bertani (LB) at 37 °C, *E. aerogenes* was grown in LB or LB containing 5% glucose and M9 salts (LBGM9) (1) medium at 34 °C, and *C. glutamicum* was grown in CM-Dex medium (2) at 31.5 °C. The following antibiotics were used for transformant selection and plasmid maintenance: 25 mg L^{-1} kanamycin in *C. glutamicum* and 40 mg L^{-1} kanamycin, 12.5 mg L^{-1} tetracycline, and 25 mg L^{-1} chloramphenicol in *E. coli* and *E. aerogenes*. All bacteria were grown with shaking in test tubes or Sakaguchi flasks capped with silicosen (Shin-Etsu Polymer, Tokyo, Japan), except for anaerobic succinate production.

Table 1. Microbial strains and plasmids used in this study

Strains and Plasmids	Description	Antibiotic resistance[a]	Reference or source
Strain			
ES06	*E. aerogenes* AJ110637Δ*adhE*Δ*ldhA*Δ*pta*Δ*budA*Δ*poxB::Ptac-pckA* (*A. succinogenes*)	Km	(3)
FK01	*E. aerogenes* ES06Δ*sdhA*Δ*yeeA*	None	This work
FK02	*E. aerogenes* FK01Δ*yjjPB*	None	This work
FK03	*E. aerogenes* FK01/pSTV28	Cm	This work
FK04	*E. aerogenes* FK02/pSTV28	Cm	This work
FK05	*E. aerogenes* FK01/pFK18	Cm	This work
FK06	*E. aerogenes* FK02/pFK18	Cm	This work
FK07	*E. aerogenes* ES06Δ*sdhA*Δ*yeeA*/RSFPP	Tet	This work
FK08	*E. aerogenes* EK07/pSTV28	Cm	This work
FK09	*E. aerogenes* EK07/pFK18	Cm	This work
FK10	*E. aerogenes* EK07/pFK19	Cm	This work
FK11	*E. aerogenes* EK07/pFK20	Cm	This work
AJ110655	*C. glutamicum* MJ233Δ*ldh*	None	(4)
FK12	*C. glutamicum* AJ110655/pVK9	Km	(4)
FK13	*C. glutamicum* MJ233Δ*ldh*Δ*sucE1*	None	(4)
FK14	*C. glutamicum* FK13/pVK9	Km	(4)
FK15	*C. glutamicum* FK13/pFK21	Km	This work
FK16	*C. glutamicum* FK13/pFK22	Km	This work
FK17	*C. glutamicum* FK13/pFK23	Km	This work
Plasmids			
pSTV28	Plasmid vector with the replication origin of pACYC184	Cm	This work
pFK18	pTSV28 containing the *E. aerogenes yjjPB* genes under control of the native promoter	Cm	This work
pFK19	pTSV28 containing the *E. aerogenes yjjP* gene under control of the native promoter	Cm	This work
pFK20	pTSV28 containing the *E. aerogenes yjjB* gene under control of the *tac* promoter	Cm	This work

RSFPP	Plasmid for expression of the *E. coli prpC* and *ppc* genes containing the RSF1010 origin of replication	Tet	This work
pRSFRedIX	Broad-host-range λ Red expressing plasmid	Cm	(5)
pMW-*attL*$_\lambda$-Km^R-*attR*$_\lambda$	Cassette for gene disruption containing a kanamycin resistance gene	Km	(6)
pVK9	*E. coli* and *C. glutamicum* shuttle vector	Km	(7,8)
pFK21	pVK9 containing the *E. aerogenes yjjPB* genes under control of the *msrA* promoter	Km	This work
pFK22	pVK9 containing the *E. aerogenes yjjP* gene under control of the *msrA* promoter	Km	This work
pFK23	pVK9 containing the *E. aerogenes yjjB* gene under control of the *msrA* promoter	Km	This work

[a]Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol; Tet, tetracycline

Plasmid construction. To construct plasmids pFK18 and pFK19, in which the genes are expressed under their native promoters, the *EayjjPB* and *EayjjP* genes were PCR amplified using chromosomal DNA from *E. aerogenes* AJ110637 (9) with primers EayjjPB_F/EayjjPB_R and EayjjPB_F/EayjjP_R, respectively, and PrimeSTAR MaDNA Polymerase (TAKARA BIO INC., Shiga, Japan). The chromosomal DNA of all bacteria was extracted using the PurElute Bacterial Genomic Kit (EdgeBio, Gaithersburg, MD, USA). The amplified DNA fragments were inserted into the *Bam*HI and *Pst*I sites in plasmid pSTV28 using the In-Fusion HD Cloning Kit (TAKARA BIO). To construct pFK20 plasmid, in which the *yjjB* gene is expressed under the *tac* promoter (because *yjjPB* form an operon), the *tac* promoter was amplified using vector pTWV-P_{tac}-T_{trp} (10) as the DNA template with primers AeyjjB_P1/AeyjjB_P2 and the *EayjjB* gene was amplified using *E. aerogenes* AJ110637 chromosomal DNA with

primers EayjjB-P3/EayjjPB-R. The amplified DNA fragments were inserted into the *Bam*HI and *Pst*I sites in plasmid pSTV28 using the In-Fusion HD Cloning Kit.

Plasmid RSFPP was constructed from plasmid RSFPPG (11), which carries *E. coli prpC*, *ppc*, and *gdhA*. Plasmid RSFPPG was digested with *Nsp*V and then self-ligated resulting in plasmid RSFPP in which *gdhA* is deleted.

To construct plasmids pFK21, pFK22, and pFK23, the *msrA* promoter was amplified from the chromosomal DNA of *C. glutamicum* AJ13869 (7) with primers CEayjjPB-P1/CEayjjPB-P2. The *EayjjPB*, *EayjjP*, and *EayjjB* genes were amplified from the chromosomal DNA of *E. aerogenes* AJ110637 with primers CEayjjPB-P3/CEayjjPB-P4, CEayjjPB-P3/CEayjjP-P4, and CEayjjB-P3/CEayjjPB-P4, respectively. Next, the *msrA* promoter and the corresponding gene fragment were inserted into *Bam*HI- and *Pst*I- digested plasmid pVK9 (7) using the In-Fusion HD Cloning Kit. pVK9 is an *E. coli–C. glutamicum* shuttle vector obtained by integration of the *ori* region of plasmid pHM1519 (8) into plasmid pHSG299 (TAKARA BIO). All primer sequences are listed in Table 2.

Disruption of the *EayjjPB* and *yeeA* genes in *E. aerogenes*. *EayjjPB* and *yeeA* were disrupted using the λ Red gene knockout system (6,12) with the Red-recombineering helper plasmid pRSFRedIX (5). pRSFRedIX carries the λ-Red *γ*, *β*, and *exo* genes downstream of a *lac* promoter, the *int* and *xis* genes downstream of the *araB* promoter, and the *Bacillus subtilis sacB* gene (encoding levansucrase) downstream of the *lac* promoter; this allows for efficient recovery of this plasmid from cells grown in a sucrose-containing medium. Using pRSFRedIX, integration of the λ-Red system by IPTG induction and the subsequent removal of the antibiotic resistance gene with the λ Int/Xis-driven system by arabinose induction can be performed without any other helper

plasmid. A removable kanamycin resistance gene flanked by *attL*$_\lambda$ and *attR*$_\lambda$ was amplified with primers ΔyjjPB_F/ΔyjjPB_R and ΔyeeA_F/ΔyeeA_R respectively, and using plasmid pMW-*attL*$_\lambda$-Km^R-*attR*$_\lambda$ as the DNA template (6). Antibiotic resistant transformants were designated as strain Δ*EayjjPB*::Km and Δ*yeeA*::Km, respectively. To remove the kanamycin resistance gene from the genetically modified bacterial chromosome, the strains were spread on LB plates containing 40 mg L^{-1} chloramphenicol and 10 mM L-arabinose. Clones that formed single colonies were identified and elimination of the kanamycin resistance gene was confirmed by growth on LB medium containing 50 mg L^{-1} kanamycin. Strains that could not grow on kanamycin plates were utilized as kanamycin marker-free strains. To remove pRSFRedIX from the marker-free strains, the strains were spread on LB plates containing 10% sucrose and 1 mM IPTG to obtain single colonies. Removal of pRSFRedIX was confirmed on LB plates containing 40 mg L^{-1} chloramphenicol. Strains in which pRSFRedIX was removed were designated as strains Δ*yjjPB* and Δ*yeeA*, respectively. All primer sequences are listed in Table 2.

Table 2. Primers used in this study

Primer	***Sequence (5′–3′)***
AeyjjPB_F	GGCCAGTGCCAAGCTCGCGCCAATAATGGCAACCTG
AeyjjPB_R	CGGTACCCGGGGATCAATTCGTGAAGACATAACGC
AeyjjP_R	CGGTACCCGGGGATCTGGGACGTTGAACACCATCG
AeyjjB_P1	GGCCAGTGCCAAGCTCCCCCTGTGGCAAATTAATC
AeyjjB_P2	TGCCCATCCGCGTAGTCCTGTGTGAAATTGTTATC
AeyjjB_P3	CTACGCGGATGGGCATAATC
ΔyjjPB_F	GGGTGGAGTTCAGGTACAAGTATCAATAGAGACGCGGACAGCAGGTACT TTGAAGCCTGCTTTTTTATACTAAGTTGGCA
ΔyjjPB_R	GCGCTGGCGGTTACACGCGCGGGCGTTTGCGATAGAGCCACAGACCGGG ACGCTCAAGTTAGTATAAAAAAGCTGAACGA
ΔyeeA_F	GATCCGTCTGTATCGTCATTACCGAATTGTGCACGGTATCCGCATCGCGCT GAAGCCTGCTTTTTTATACTAAGTTGGCA
ΔyeeA_R	GCCAGCTCAATGGTCAGCCAAACATAGCCGTGAATCGGCGTTTCCGACA GCGCTCAAGTTAGTATAAAAAAGCTGAACGA
CAeyjjPB-P1	CCAAGCTTGCATGCCATTTGCGCCTGCAACGTAGGTTG
CAeyjjPB-P2	CGCTGCTCTGATTTATCTGCCTGCATAACAGGAATGTTCCTTTCGAAAA
CAeyjjPB-P3	TTTTCGAAAGGAACATTCCTGTTATGCAGGCAGATAAATCAGAGCAGCG
CAeyjjPB-P4	CGGTACCCGGGGATCAATTCGTGAAGACATAACGC
CyjjP-P4	CGGTACCCGGGGATCTGGGACGTTGAACACCATCG
CyjjB-P2	CGAAAAGATAAGAGATTATGCCCATAACAGGAATGTTCCTTTCGAAAA
CyjjB-P3	TTTTCGAAAGGAACATTCCTGTTATGGGCATAATCTCTTATCTTTTCG

Succinate fermentation in test tubes under aerobic conditions. Pre-culturing for cell growth was performed on LBGM9 plates at 34 °C for 16 h. Three loops of the pre-cultured cells were inoculated using a 1 μL inoculating loop (Thermo Fisher Scientific K.K., Kanagawa, Japan) into test tubes containing 5 mL succinate fermentation medium [30 g L^{-1} sucrose, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 g L^{-1} yeast extract, 5 g L^{-1} $(NH_4)_2SO_4 \cdot 7H_2O$, 2 g L^{-1} KH_2PO_4, 10 mg L^{-1} $MnSO_4 \cdot 5H_2O$, and 10 mg L^{-1}

$FeSO_4 \cdot 7H_2O$] with 20 g L^{-1} precipitated $CaCO_3$ sterilized by dry heat at 180 °C for 3 h (Japanese Pharmacopoeia, Tokyo, Japan). Following inoculation, succinate fermentation was performed at 34 °C and a rotation speed of 120 rpm for 18 h. The formula used to calculate the dry cell weight (DCW) of *E. aerogenes* was: [DCW]=29.1 × (OD_{600}) / 100 (g, L^{-1}) (13).

Succinate fermentation in 1.5 mL microfuge tubes under anaerobic conditions. To evaluate succinate production under anaerobic conditions, I employed a convenient evaluation system utilizing 1.5 mL microfuge tubes (3,4,9,13). *E. aerogenes* was pre-cultured on LBGM9 plates at 34 °C for 18 h. The pre-cultured cells were washed twice with cold anaerobic medium without $CaCO_3$ and then inoculated into 1.5 mL microfuge tubes containing 1.2 mL of anaerobic medium [40 g L^{-1} glucose, 1 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 g L^{-1} Bacto yeast extract, 1 g L^{-1} $(NH_4)_2SO_4 \cdot 7H_2O$, 1 g L^{-1} KH_2PO_4, 10 mg L^{-1} $MnSO_4 \cdot 5H_2O$, and 10 mg L^{-1} $FeSO_4 \cdot 7H_2O$; pH 7.0 with KOH] with 50 g L^{-1} precipitated $CaCO_3$ sterilized by dry heat at 180 °C for 3 h (Japanese Pharmacopoeia). After tightly capping the tubes, succinate fermentation was performed using an Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany) at 34 °C and a rotation speed of 1,400 rpm for approximately 24 h under aerobic conditions. Initial biomass was adjusted to an optical density (OD_{620}) of approximately 15. *C. glutamicum* pre-cultured on CM-Dex plates was inoculated into 3 mL 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, 20 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 20 mg L^{-1} $MnSO_4 \cdot 7H_2O$, 200 μg L^{-1} biotin, 200 μg L^{-1} thiamine·HCl (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 approximately 16 h under aerobic conditions. For succinate production, 250 μL of the pre-culture was collected

and immediately mixed with 250 μL SA2 medium [100 g L^{-1} glucose, 15 g L^{-1} sodium sulfite, and 71.5 g L^{-1} $MgCO_3$ (separately sterilized)] in a microfuge tube, followed by shaking at 32 °C for approximately 48 h under anaerobic conditions. The formula used to calculate the DCW of *C. glutamicum* was: [DCW]=22.707 × (OD_{620}) / 100 (g, L^{-1}) (2).

Metabolite analysis. Organic acids that accumulated in the medium were analyzed by high-performance liquid chromatography (HPLC) using a CDD-10AD system (Shimadzu Co. Ltd., Kyoto, Japan) with a suitable dilution, as described in the author's previous report (3,4,9,13). Glucose concentrations were analyzed using an AS-310 Biotech Analyzer (Sakura SI Co. Ltd., Tokyo, Japan). OD was measured at 620 nm with a U-2001 spectrometer (Hitachi Co. Ltd, Tokyo, Japan). Broth containing $CaCO_3$ or $MgCO_3$ was diluted with 0.1 N HCl prior to measuring OD_{620}.

Results

Construction of an aerobic and anaerobic succinate producer. In my recent report, the split poly-peptides YjjP and YjjB (YjjPB) were genetically predicted to form a novel succinate exporter in *E. coli*. *E. aerogenes* possesses homologs of YjjP and YjjB with 83% and 81% identity, respectively (14). Phylogenic analysis of YjjP and YjjB amino acid sequences showed that *E. aerogenes* YjjP and YjjB (EaYjjP and EaYjjB) were closer to *E. coli* YjjP and YjjB than to *M. succiniciproducens* and *A. succinogenes* (Fig. 1A). EaYjjP and EaYjjB consist of 258 and 157 amino acids, respectively. A conserved domain search using the Pfam database (15) indicated that EaYjjP contains a ThrE domain and EaYjjB has a ThrE_2 domain in their respective peptide sequences (Fig. 1B and C). ThrE and ThrE_2 domains are conserved in the ThrE family, which consists of bacterial and archaeal proteins that catalyze the export of L-threonine from the cell (15). In addition, hydropathy analysis (Fig. 1D) using the SOSUI prediction program (16), suggested that EaYjjP and EaYjjB possess five and four α-helical transmembrane domains, respectively. These results indicate that EaYjjPB might be multi α-helical inner membrane proteins.

E. aerogenes has been established as a good strain for succinate production under anaerobic conditions (3,13). Furthermore, it is known that succinate is produced under aerobic conditions in a succinate dehydrogenase deletion mutant (17) and that this deletion barely affects succinate production levels under anaerobic conditions (18), as the conversion of fumarate to succinate is catalyzed by fumarate reductase and not succinate dehydrogenase under anaerobic conditions (13). However, it is possible that produced succinate could be converted into fumarate by succinate dehydrogenase under

anaerobic conditions. Therefore, in order to examine the contribution of *EayjjPB* to succinate production in *E. aerogenes* under aerobic and anaerobic conditions, strains FK07 and FK01 were constructed based on the anaerobic succinate producer strain ES06 (3). In strain ES06, anaerobic by-production pathways, such as acetate, 1,4-butanediol, and ethanol, were disrupted and the *pckA* gene, encoding phosphoenolpyruvate carboxykinase, derived from *Actinobacillus succinogenes* strain 130z (19) was introduced in order to increase the flux of the anaplerotic pathway. Furthermore, in order to more clearly demonstrate the contribution of *yjjPB* to succinate production, *yeeA* was deleted because the author hypothesizes it encodes a putative dicarboxylic acid transporter (manuscripts in preparation). Strain FK07 contains plasmid RSFPP, which carries the *ppc* and *prpC* genes encoding phosphoenolpyruvate carboxylase and 2-methylcitrate synthase, respectively, in order to increase citrate supply for succinate production under aerobic conditions. The *ppc* gene was amplified in order to increase oxaloacetate supply (20,21). 2-methylcitrate synthase, encoded by *prpC*, which catalyzes the Claisen condensation of propionyl-CoA with oxaloacetate to convert methylcitrate, is known to also have citrate synthase activity (22,23). Therefore, *prpC* was amplified in order to increase citrate supply from oxaloacetate and acetyl-CoA.

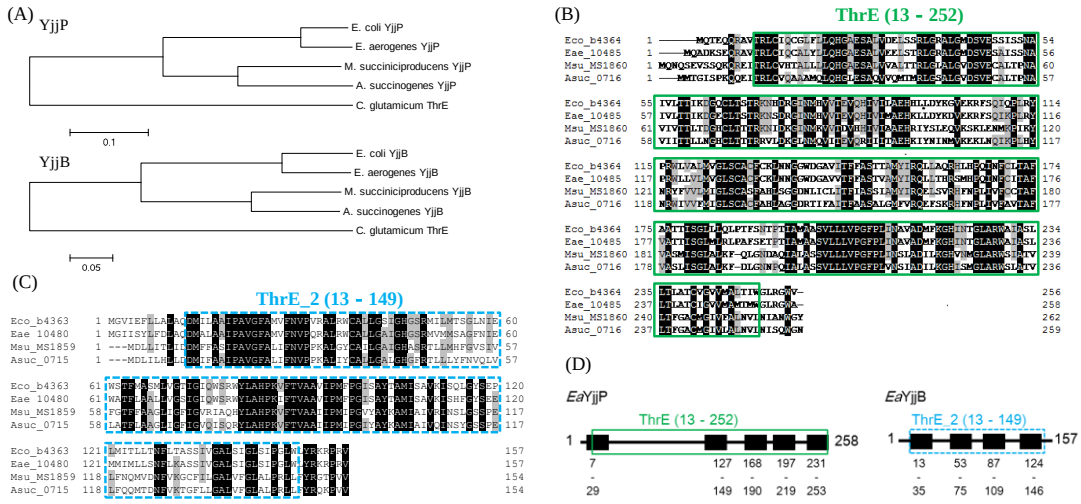

Fig. 1 Phylogenic analysis and multiple alignments of YjjP and YjjB.

(A) The phylogenic tree was constructed using the neighbor joining method with the MEGA6 program (30). Numbers and branch lengths indicate the proportion of different sites (p-distance). Multiple alignments of (B) YjjP and (C) YjjB) performed using the GENETYX software. (D) The transmembrane helices and conserved domains of EaYjjPB. The SOSUI transmembrane helix prediction program (15) and the Pfam database (16) were used for model construction. The putative transmembrane helices are indicated by closed boxes and the conserved domains by stippled boxes.

All sequence data were obtained from the GenBank database using the following accession numbers: *Eco_b4364*, *E. coli* NP_418784; *Eae_10485*, *E. aerogenes* YP_004592292; Msu_MS1860, *M. succiniciproducens* AAU38467; Asuc_0716, *A. succinogenes* ABR74089; *Eco_b4363*, *E. coli* NP_418783; *Eae_10480*, *E. aerogenes* YP _004592291; Msu_MS1859, *M. succiniciproducens* AAU38466; Asuc_0715, *A. succinogenes* ABR74088; and *C. glutamicum* ThrE, NP_601823.

Effect of *EayjjP, EayjjB* or *EayjjPB* expression on succinate production under aerobic conditions. The effects of *EayjjP*, *EayjjB*, or *EayjjPB* expression on succinate production under aerobic conditions were evaluated using strain FK07 as an aerobic succinate producer. As shown in Fig. 2A, the production level of succinate was 4.1 g L^{-1} for strain FK08 (FK07/pSTV28) and 10 g L^{-1} for strain FK09 (FK07/pFK18). Expression of *EayjjPB* increased the cell mass 1.4-fold, from 3.3 g L^{-1} in strain FK08 to 4.5 g L^{-1} in strain FK09. Succinate specific productivity was 1.3 (g-succinate/g-cell mass) for strain FK08 and 2.2 for strain FK09 (Fig. 2B). Furthermore, the level of pyruvate, a major by-product of aerobic succinate production in *E. aerogene*s, decreased significantly from 4.0 g L^{-1} in FK08 to below the detection limit in FK09. Fumarate, malate, and acetate, which are major by-products in anaerobic succinate production, were barely detected (data not shown). In addition, expression of *EayjjP* or *EayjjB* independently, did not lead to significant changes in the production levels of succinate and pyruvate (FK10 and FK11 in Fig. 2A). These results show that co-expression of *EayjjPB* has a significant effect on succinate production in *E. aerogenes* under aerobic conditions and that both genes are required for aerobic succinate production.

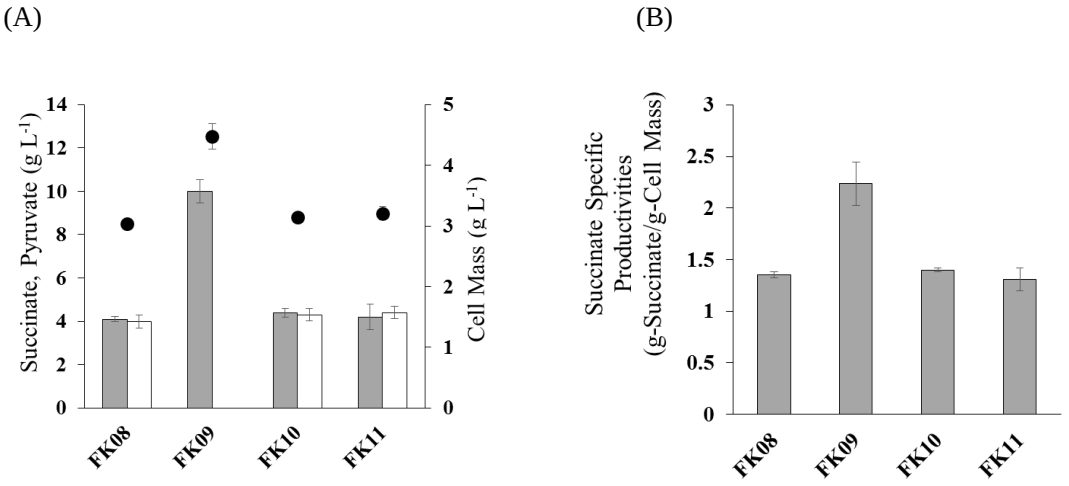

Fig. 2 Effect of *EayjjP*, *EayjjB*, or *EayjjPB* expression on succinate production in *E. aerogenes* under aerobic conditions.

Strain FK07 was used as the host. *E. aerogenes* FK08 (control – FK07/pSTV28), FK09 (FK07/pFK18), FK10 (FK07/pFK19), and FK11 (FK07/pFK20) were used. Succinate production was performed in test tubes at 34 °C and a rotation speed of 120 rpm for 18 h under aerobic conditions, as described in the "Materials and Methods" section. (A) Cell Mass (closed circles), succinate concentration (gray columns), and pyruvate concentration (white columns). (B) Succinate specific productivity (g-succinate/g-cell mass). Data from three independent experiments are shown as mean ± SE.

Effect of *EayjjPB* expression and deletion on succinate production under anaerobic conditions. Subsequently, I examined succinate production in an *EayjjPB*-expressing strain and an *EayjjPB* deletion mutant under anaerobic conditions. Strain FK01 was used as an anaerobic succinate producer. As shown in Fig. 3A, the

succinate production level was 5.7 g L^{-1} in strain FK03 (FK01/pSTV28) and 5.8 g L^{-1} in strain FK05 (FK01/pFK18). However, expression of *EayjjPB* resulted in an increase in succinate yield from 53% to 60% (Fig. 3A), as the consumed glucose level was decreased by the expression of *EayjjPB* (data not shown). In contrast, succinate specific productivity (g-succinate/g-cell mass) decreased from 0.74 for strain FK03 to 0.69 for strain FK05 (Fig. 2C). Furthermore, the production levels of fumarate and malate, which are intermediates of the succinate-biosynthesis pathway, increased following *EayjjPB* expression from 0.63 g L^{-1} to 1.3 g L^{-1} and from 0.21 g L^{-1} to 0.34 g L^{-1}, respectively (Fig. 3B). The level of acetate, one of the major by-products, decreased from 0.72 g L^{-1} to 0.43 g L^{-1} (Fig. 3B). Succinate accumulation, yield, and succinate specific productivity were 4.3 g L^{-1}, 48%, and 0.62, respectively, for strain FK04 (FK01Δ*yjjPB*/pSTV28); succinate accumulation, yield, and succinate specific productivity all decreased when *yjjPB* were deleted. Malate was not detected in strain FK04. Furthermore, succinate accumulation, yield, and succinate specific productivity were 5.4 g L^{-1}, 59%, and 0.69, respectively, for strain FK06 (*EayjjPB* complementation), comparable to the levels of strain FK05 (Fig. 3A), suggesting that pFK18 expressed *EayjjPB* at a much higher level than endogenous *EayjjPB* present on the chromosome. These results demonstrate that YjjPB mainly contribute to enhancing not only succinate, but also fumarate and malate production under anaerobic conditions.

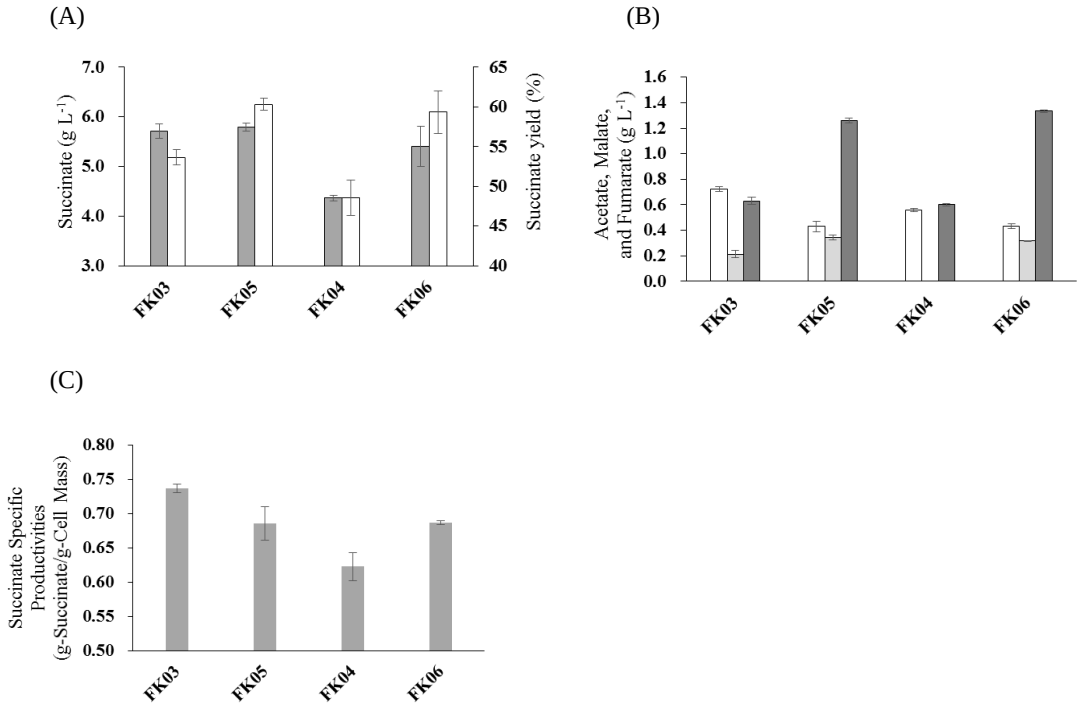

Fig. 3 Effect of *EayjjPB* expression and deletion on succinate production in *E. aerogenes* under anaerobic conditions.

FK01 strain was used as the host. *E. aerogenes* FK03 (control – FK01/pSTV28), FK05 (FK01/pFK18), FK04 (FK01Δ*EayjjPB*/pSTV28), and FK06 (FK01 Δ*EayjjPB*/pFK18) were used. Succinate production was performed in 1.5 mL microfuge tubes at 34 °C and a rotation speed of 1,400 rpm for 24 h under anaerobic conditions, as described in the "Materials and Methods" section. (A) Succinate concentration (gray columns) and succinate yield (white columns). Succinate yield was calculated as the amount of produced succinate relative to consumed glucose (g/g). (B) Acetate concentration (white columns), malate concentration (light gray columns), and fumarate concentration (dark gray columns). (C) Succinate specific productivity (g-succinate/g-cell mass). Data from three independent experiments are shown as mean ± SE.

Complementation of *sucE1* deletion with *EayjjP, EayjjB,* and *EayjjPB* in *C. glutamicum*. Previously, using partially purified histidine-tagged SucE1 the author demonstrated that SucE1 is a succinate exporter in *C. glutamicum* with succinate counterflow and self-exchange activities. In addition, I showed that the *sucE1*-deletion mutant was unable to produce succinate because it lacked a succinate export carrier and that complementation of the *sucE1* gene by plasmid transformation restored succinate production under these conditions (4). Therefore, I next examined whether succinate production could be complemented by introducing *EayjjPB*, *EayjjP*, or *EayjjB* into *C. glutamicum* strain FK13, which cannot produce succinate because of a disruption of the succinate exporter, SucE1. Fig. 4 shows the results for succinate fermentation conducted in 1.5 mL microfuge tubes under anaerobic conditions. Strain FK12 (control – AJ110655/pVK9) produced 50 g L^{-1} succinate, while FK14 (AJ110655Δ*sucE1*/pVK9) produced only 1.0 g L^{-1} succinate. FK16 (AJ110655Δ*sucE1*/pFK22) and FK17 (AJ110655Δ*sucE1*/pFK23) produced 1.5 and 1.3 g L^{-1} succinate, respectively (Fig. 4A). However, succinate production was restored to 45 g L^{-1} in strain FK15 (AJ110655Δ*sucE1*/pFK21). Succinate specific productivity (g-succinate/g-cell mass) was 9.7 for strain FK12, 0.3 for strain FK14, and 8.4 for FK15; the productivity of FK16 and FK17 was comparable to that of FK14 (Fig. 4B). These results demonstrate that expression of EaYjjPB almost completely complemented SucE1 function and that both proteins are required for complementation.

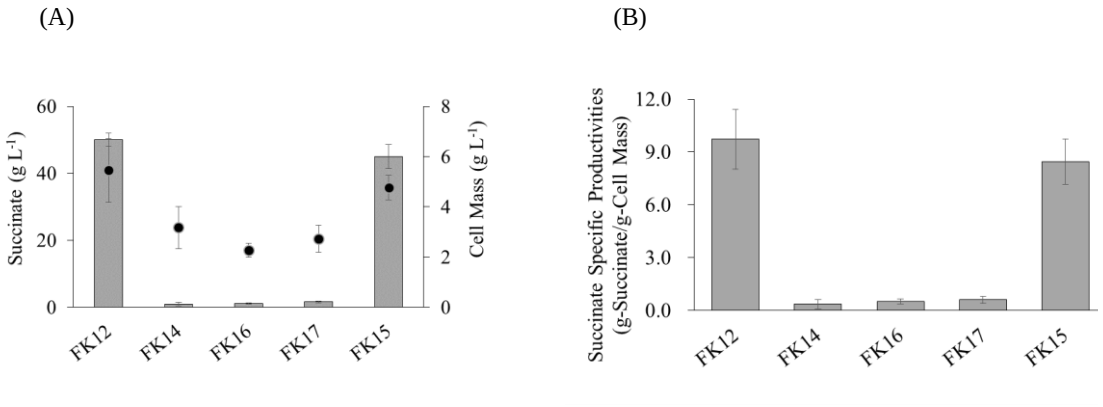

Fig. 4 Complementation of the *sucE1* deletion with *EayjjP*, *EayjjB*, and *EayjjPB* in *C. glutamicum.*

E. aerogenes yjjP and *yjjB* were introduced into *C. glutamicum* FK13, a strain in which the limiting step for succinate production is succinate export. *C. glutamicum* FK12 (control – AJ110655/pVK9), FK14 (AJ110655Δ*sucE1*/pVK9), FK16 (AJ110655Δ*sucE1*/pFK22), FK17 (AJ110655Δ*sucE1*/pFK23), and FK15 (AJ110655Δ*sucE1*/pFK21) were used. Succinate production was performed in 1.5 mL microfuge tubes at 32 °C and a rotation speed of 1,400 rpm for 48 h under anaerobic conditions, as described in the "Materials and Methods" section. (A) Cell mass (closed circles), succinate concentration (gray columns). (B) Succinate specific productivity (g-succinate/g-cell mass). Data from four independent experiments are shown as mean ± SE.

Discussion

In this study, I determined that the products of the *E. aerogenes EayjjPB* genes contribute to succinate production under both aerobic and anaerobic conditions. Furthermore, *EayjjPB* expression also increased fumarate and malate production during anaerobic succinate production in *E. aerogenes* (Fig. 3B). My results indicate that the EaYjjPB proteins participate not only in succinate export, but also in fumarate and malate export.

Recently, I demonstrated that the *yjjPB* genes of *E. coli* contribute to succinate production under aerobic and anaerobic conditions, indicating that the YjjPB proteins participate in succinate transport (14). Furthermore, I found that other gram-negative anaerobic succinate producers, such as *E. aerogenes*, *A. succinogenes* (24), and *M. succiniciproducens* (25), possess YjjPB homologs. Phylogenic analysis of the amino acid sequences of YjjP and YjjB indicated that *E. aerogenes* YjjP and YjjB were similar to *E. coli* YjjP and YjjB (Fig. 1A). A conserved protein domain search using the Pfam database (15) indicated that EaYjjP and EaYjjB contain a ThrE domain (PF06738) and ThrE_2 domain (PF12821), respectively. In addition, transmembrane prediction of EaYjjP and EaYjjB using SOSUI (16) showed these proteins have an almost identical transmembrane structure to that of *E. coli* YjjPB. Taken together, these results indicate that EaYjjPB might function as a split-type ThrE family transporter similar to *E. coli* YjjPB.

Expression of the *EayjjPB* genes during anaerobic succinate production in *E. aerogenes* led to decreased acetate and increased malate and fumarate (Fig. 3B). These results indicate that *EayjjPB* could function as a dicarboxylate transporter that transports

not only succinate but also malate and fumarate. Moreover, during anaerobic succinate production in *E. aerogenes*, FK04, which lacks *EayjjPB*, produced 4.4 g L^{-1} succinate and 0.60 g L^{-1} fumarate; malate production in FK04 was below the detection limit. FK06 (the FK04 complementation strain) produced 5.4 g L^{-1} succinate, 0.32 g L^{-1} malate, and 1.3 g L^{-1} fumarate, indicating that *EayjjPB* expression is more effective for succinate production than for fumarate or malate production. In addition, during anaerobic succinate production in *C. glutamicum*, FK14, which lacks the succinate exporter gene, *sucE1*, produced 1.0 g L^{-1} succinate, while malate and fumarate production were below the detection limit (data not shown). FK15 (the FK14 complementation strain) produced 45 g L^{-1} succinate and 0.50 g L^{-1} malate, while fumarate production was below the detection limit in this strain (data not shown). These results also indicate that *EayjjPB* expression is more effective for succinate production; this could be caused by EaYjjPB having a higher affinity for succinate than for malate or fumarate or may be due to a higher internal succinate concentration. However, as I have previously demonstrated that the internal malate concentration is higher than the succinate concentration under microaerobic conditions (4), the more likely explanation is that EaYjjPB has a higher affinity for succinate. Expression of the *sucE1* gene during anaerobic succinate production in *C. glutamicum* has been shown to increase succinate flux and decrease internal intermediates (26), resulting in decreased external intermediates such as fumarate and malate. This difference between *E. aerogenes* and *C. glutamicum* might be caused by differences in the substrate affinity of EaYjjPB and SucE1; fumarate and malate are decreased by the expression of SucE1 (relatively lower affinity for these substrates than EaYjjPB) and increased by the expression of EaYjjPB (relatively higher affinity for these substrates than SucE1). Further studies are required

to elucidate the difference in substrate specificity between SucE1 and YjjPB.

EayjjPB expression increased cell mass both under aerobic and anaerobic conditions (Fig. 2A, 4B) and succinate production was significantly enhanced under these conditions. Furthermore, pyruvate, which is a major by-product under aerobic succinate production in *E. aerogene*s, was significantly decreased by *EayjjPB* expression (Fig. 2A), suggesting that *EayjjPB* expression decreased the internal pyruvate concentration by increasing succinate flux, as well as acetate levels, during anaerobic production (Fig. 3B). Previously I showed that deletion of the succinate exporter gene *sucE1* in *C. glutamicum* resulted in decreased cell mass under anaerobic conditions, while the internal succinate concentration increased two-fold following the deletion of *sucE1* under microaerobic conditions (4). In this study, deletion of *sucE1* decreased the cell mass of *C. glutamicum* from 5.4 g L^{-1} for FK12 to 3.1 g L^{-1} for FK14 under anaerobic conditions; cell mass recovered to 4.8 g L^{-1} in FK15 expressing *EayjjPB* (Fig. 4A). These results suggest that expression of *EayjjPB* had a positive effect on cell growth under both aerobic and anaerobic conditions by decreasing the internal succinate concentration, which constitutes a limiting factor for succinate production in these strains. Moreover, these findings indicate that EaYjjPB could be very important for industrial succinate production under both aerobic and anaerobic conditions. Further studies are required to elucidate the relationship between deletion of *EayjjPB* and internal succinate concentrations under aerobic and anaerobic conditions in *E. aerogenes*.

Although succinate concentration was decreased by deleting *EayjjPB* under anaerobic condition, FK04, which lacks *EayjjPB*, still produced a significant amount of succinate. Recently I identified YeeA and YnfM as dicarboxylate transporters (data not

shown, manuscript in preparation). However, succinate production was not decreased by deleting *ynfM* in FK04 (*yeeA* and *yjjPB* deletion background). This result suggests that other unknown transporters work as succinate transporters *in E. aerogenes*; the author is currently attempting to identify these novel succinate transporters.

Recently, I developed a complementation 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 are disrupted. A previous complementation assay indicated that the combination of *E. coli* YjjP and YjjB restored only 28% succinate production (14), while in this study, the combination of *E. aerogenes* EaYjjP and EaYjjB restored 90% of succinate production (Fig. 4). These results indicate that the succinate-transport activity of *E. aerogenes* EaYjjPB might be higher than that of *E. coli* YjjPB. Kinetic analysis of purified EaYjjPB and YjjPB will be addressed in future studies. Taken together, these results suggest that EaYjjPB possesses succinate-transport activity.

In this report, I demonstrated that *E. coli* YjjPB as well as *E. aerogenes* EaYjjPB participates in succinate transport, which strongly suggests that YjjPB might act as the major succinate transporter involved in succinate export in many gram-negative bacteria. As described in the introduction, succinate production at weakly acidic pH is considered a feasible approach for reducing total costs by limiting the use of alkali and acid in the fermentation and recovery processes (27,28). *E. aerogenes* strain G243 is much more resistant to acidic stress than *E. coli* (9), indicating that *E. aerogenes* strain G243 is a superior producer of succinate in terms of industrial application. Therefore, it is important to demonstrate that the expression of *EayjjPB* is effective for succinate production in *E. aerogenes*. Furthermore, unlike *E.coli* YjjPB, *E. aerogenes* EaYjjPB

was shown to be involved not only in succinate transport, but also in fumarate and malate transport. Therefore, it is also meaningful to demonstrate that *EayjjPB* encodes a dicarboxylate transporter. I hypothesize that fermentative succinate production could be greatly improved by using this succinate transporter.

ThrE family transporters catalyze the proton-linked transport of L-threonine or L-serine (29), whereas *E. coli* YjjPB (14) and *E. aerogenes* EaYjjPB (in this study) are putative succinate and dicarboxylate transporters, respectively. In the phylogenetic tree of the ThrE family, the gram-negative bacterial systems form one cluster (29) and all gram-negative bacterial systems are split systems derived from two adjacently mapped genes. Importantly, this study suggests the existence of variations in substrate specificity within the ThrE family transporters.

REFERENCES

1. **Sambrook, J. and Russell, D. W.:** Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor, NY (2001).
2. **Nakamura, J., Hirano, S., Ito, H., and Wach, M.:** Mutations of the *Corynebacterium glutamicum* NCgl1221 gene, encoding a mechanosensitive channel homolog, induce L-glutamic acid production, Appl. Environ. Microbiol., **73**, 4491–4498 (2007).
3. **Tajima, Y., Yamamoto, Y., Fukui, K., Nishio, Y., Hashiguchi, K., Usuda, Y., and Sode, K.:** Impact of an energy-conserving strategy on succinate production under weak acidic and anaerobic condition in *Enterobacter aerogenes*, Microb. Cell Fact., **14**, 80 (2015).
4. **Fukui, K., Koseki, C., Yamamoto, Y., Nakamura, J., Sasahara, A., Yuji, R., Hashiguchi, K., Usuda, Y., Matsui, K., Kojima, H., and Abe, K.:** Identification of succinate exporter in *Corynebacterium glutamicum* and its physiological roles under anaerobic conditions, J. Biotechnol., **154**, 25–34 (2011).
5. **Yamakami, S., Hara, Y., Fukui, K., and Koizumi, N.:** Method for producing useful substance, International patent WO2015005405 A1 (2015).
6. **Katashkina, J. I., Hara, Y., Golubeva, L. I., Andreeva, I. G., Kuvaeva, T. M., and Mashko, S. V.:** Use of the lambda Red-recombineering method for genetic engineering of *Pantoea ananatis*, BMC Mol. Biol., **10**, 34 (2009).
7. **Chinen, A., Kozlov, Y. I., Hara, Y., Izui, H., and Yasueda, H.:** Innovative metabolic pathway design for efficient L-glutamate production by suppressing CO_2 emission, J. Biosci. Bioeng., **103**, 262–269 (2007).

8. **Miwa, K., Matsui, K., Terabe, M., Ito, K., Ishida, M., Takagi, H., Nakamori, S., and Sano, K.:** Construction of novel shuttle vectors and a cosmid vector for the glutamic acid-producing bacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*, Gene, **39**, 281–286 (1985).
9. **Tajima, Y., Kaida, K., Hayakawa, A., Fukui, K., Nishio, Y., Hashiguchi, K., Fudou, R., Matsui, K., Usuda, Y., and Sode, K.:** Study of the role of anaerobic metabolism in succinate production by *Enterobacter aerogenes*, Appl. Microbiol. Biotechnol., **98**, 7803–7813 (2014).
10. **Nishio, Y., Ogishima, S., Ichikawa, M., Yamada, Y., Usuda, Y., Masuda, T. and Tanaka, H.:** Analysis of L-glutamic acid fermentation by using a dynamic metabolic simulation model of *Escherichia coli*, BMC Syst. Biol., **7**, 92–103 (2013).
11. **Takikawa, R., Hara, Y., Nonaka, G. and Takumi, K.:** L-amino acid-producing microorganism and a method for producing an L-amino acid, U.S. patent 8206954 (2012).
12. **Datsenko, K. A. and Wanner, B. L.:** One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products, Proc. Natl. Acad. Sci. USA, **97**, 6640–6645 (2000).
13. **Tajima, Y., Yamamoto, Y., Fukui, K., Nishio, Y., Hashiguchi, K., Usuda, Y., and Sode, K.:** Effects of eliminating pyruvate node pathways, and co-expression of heterogeneous carboxylation enzymes, on succinate production by *Enterobacter aerogenes*, Appl. Environ. Microbiol., **81**, 929–937 (2015).
14. **Fukui, K., Nanatani, K., Hara, Y., Yamakami, S., Yahagi, D., Chinen, A., Tokura, M., and Abe, K.:** *Escherichia coli yjjPB* genes encode a succinate transporter important for succinate production, Biosci. Biotechnol. Biochem., **4**, 1–8

(2017).

15. **Finn, R. D., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Mistry, J., Mitchell, A. L., Potter, S. C., Punta, M., Qureshi, M., Sangrador-Vegas, A., Salazar, G. A., Tate, J., and Bateman, A.:** The Pfam protein families database: towards a more sustainable future, Nucleic Acids Res., **44**, D279–285 (2016).
16. **Hirokawa, T., Boon-Chieng, S., and Mitaku, S.:** SOSUI: classification and secondary structure prediction system for membrane proteins, Bioinformatics, **14**, 378–379 (1998).
17. **Lin, H., Bennett, G. N., and San, K-Y.:** Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate, Biotechnol. Bioeng., **20**, 148–156 (2005).
18. **Steinsiek, S., Frixel, S., Stagge, S., SUMO, and Bettenbrock, K.:** Characterization of *E. coli* MG1655 and *frdA* and *sdhC* mutants at various aerobiosis levels, J. Biotechnol., **154**, 35–45 (2011).
19. **Van der Werf, M. J., Guettler, M. V., Jain, M. K., and Zeikus, J. G.:** Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus sp.* 130Z, Arch. Microbiol., **167**, 332–342 (1997).
20. **Millard, C. S., Chao, Y. P., Liao, J. C., and Donnelly, M. I.:** Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in *Escherichia coli*, Appl. Environ. Microbiol., **62**, 1808–1810 (1996).
21. **Lin, H., San, K. Y., and Bennett, G. N.:** Effect of *Sorghum vulgare* phosphoenolpyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*, Appl.

Microbiol. Biotechnol., **67**, 515–523 (2005).

22. **Textor, S., Wendisch, V. F., De Graaf, A. A., Muller, U., Linder, M. I., Linder, D., and Buckel, W.:** Propionate oxidation in *Escherichia coli*: evidence for operation of a methylcitrate cycle in bacteria, Arch. Microbiol., **168**, 428–436 (1997).
23. **Gerike, U., Hough, D. W., Russell, N. J., Dyall-Smith, M. L., and Danson, M. J.:** Citrate synthase and 2-methylcitrate synthase: structural, functional and evolutionary relationships, Microbiology, **144**, 929–935 (1998).
24. **Guettler, M. V., Rumler, D., and Jain, M. K.:** *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen, Int. J. Syst. Evol. Microbiol., **49**, 207–216 (1999).
25. **Lee, P. C., Lee, S. Y., Hong, S. H., and Chang, H. N.:** Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen, Appl. Microbiol. Biotechnol., **58**, 663–668 (2002).
26. **Zhu, N., Xia, H., Yang, J., Zhao, X., and Chen, T.:** Improved succinate production in *Corynebacterium glutamicum* by engineering glyoxylate pathway and succinate export system, Biotechnol. Lett., **36**, 553–560 (2014).
27. **Cheng, K-K., Zhao, X-B., Zeng, J., Wu, R-C., Xu, Y-Z., Liu, D-H., and Zhang, J-A.:** Downstream processing of biotechnological produced succinic acid, Appl. Microbiol. Biotechnol., **95**, 841–850 (2012).
28. **Li, Q., Wang, D., Wu, Y., Li, W-L., Zhang, Y-J., Xing, J-M., and Su, Z-G.:** One step recovery of succinic acid from fermentation broths by crystallization, Sep. Purif. Technol., **72**, 294–300 (2010).
29. **Yen, M. R., Tseng, Y. H., Simic, P., Sahm, H., Eggeling, L., and Saier, M. H.:**

The ubiquitous ThrE family of putative transmembrane amino acid efflux transporters, Res. Microbiol., **153**, 19–25 (2002).

30. **Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S.:** MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0., Mol. Biol. Evol., **30**, 2725–2729 (2013).

Summary

Enterobacter aerogenes, a gram-negative, rod-shaped bacterium, is an effective producer of succinate from glucose via the reductive tricarboxylic acid cycle under anaerobic conditions. However, to date, succinate-exporter genes have not been identified in *E. aerogenes*, although succinate exporters have a large impact on fermentative succinate production. Recently, the author genetically identified *yjjP and yjjB*, as genes encoding a succinate transporter in *Escherichia coli*. Evaluation of the *yjjPB* homologs in *E. aerogenes* (*EayjjPB*) showed that succinate accumulation increased from 4.1 g L^{-1} to 9.1 g L^{-1} when the *EayjjPB* genes were expressed under aerobic conditions. Under anaerobic conditions, succinate yield increased from 53% to 60% by *EayjjPB* expression and decreased to 48% by deletion of *EayjjPB*. Furthermore, the production levels of fumarate and malate, which are intermediates of the succinate-biosynthesis pathway, were also increased by *EayjjPB* expression. A complementation assay conducted in *Corynebacterium glutamicum* strain AJ110655Δ*sucE*1 demonstrated that both EaYjjP and EaYjjB are required for the restoration of succinate production. Taken together, these results suggest that EaYjjPB constitute as a dicarboxylate transporter in *E. aerogenes* and that the products of both genes are required for dicarboxylate transport.

CHAPTER III

Identification of *ynfM* gene encoding dicarboxylate transporter in *Corynebacterium glutamicum* applicable to succinate production

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. Recently, is was 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. *C. glutamicum* has a gene encoding a protein (CgYnfM) homologous to PaYnfM. This fact motivated me 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, I 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 (1). *E. coli* cells were grown in Luria–Bertani (LB) broth (1) at 37ºC. *C. glutamicum* cells were grown in CM-Dex medium (2) at 31.5ºC. Kanamycin was used at 25 or 40 mg L^{-1} to select for transformants and maintain plasmids in *C. glutamicum* and *E. coli*, respectively. 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.

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmid	Description	Antibiotic resistance [1]	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	(3)
FK948	*Corynebacterium glutamicum* AJ110655Δ*sucE1*	None	(3)
FK902	*Corynebacterium glutamicum* AJ110655/pVK9	Km	(3)
FKS3	*Corynebacterium glutamicum* FK948/pVK9	Km	(3)
FKS34	*Corynebacterium glutamicum* FK948/pVK9::PmsrA-CgynfM	Km	This work
FKS35	*Corynebacterium glutamicum* FK948/pVK9::PmsrA-sucE1	Km	(3)
FK15	*Corynebacterium glutamicum* FK948/pVK9::PmsrA-AeyjjP	Km	(4)
Plasmid			
pVK9	An *Escherichia coli* and *Corynebacterium glutamicum* shuttle vector	Km	(5)
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	(3)
pVK9::PmsrA-AeyjjPB	pVK9 containing the *Enterobacter aerogenes AeyjjPB* gene under control of the *msrA* promoter	Km	(4)

[1]Km, kanamycin;

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 (5) 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 (5) 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 (6) into plasmid pHSG299 (Takara Bio).

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*	GCATATGTATGACACCGTCAAAGGTTCCGACTACATCGGTGACCAGGACG TGAAGCCTGCTTTTTTATACTAAGTTGGCA
Δ*sdhA-attR*	TCCAGCTCAAGGCACTCAATACGCTGTGTATTGAAGTCAGGTGAGCGGTC CGCTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ppc-F	TCAGGTGTGTTTAAAGCTGTTCTGCTGGGCAATACCCTGCAGTTTCGGGTC GCTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ppc-R	GTGAAGGATACAGGGCTATCAAACGATAAGATGGGGTGTCTGGGGTAATT GAAGCCTGCTTTTTTATACTAAGTTGGCA
Ptac-sucCD-F	TTACTGCTGCTGTGCAGACTGAATCGCAGTCAGCGCGATGGTGTAGACGA CGCTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-sucCD-R	CCGCCATATGAACGGCGGGTTAAAATATTTACAACTTAGCAATCAACCATG AAGCCTGCTTTTTTATACTAAGTTGGCA
Ptac-ybjL-F	TTACCTTAGCCAGTTTGTTTTCGCCAGTTCGATCACTTCATCACCGCGTCC GCTCAAGTTAGTATAAAAAAGCTGAACGA
Ptac-ybjL-R	ACACTAGTAAAATATATTGTTACTTTACTATCGTTTAGGTGCGCTGAATTGA AGCCTGCTTTTTTATACTAAGTTGGCA

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 ΔsdhA-1/ΔsdhA-2 primer pair, whereas the C-terminal fragment was amplified using the ΔsdhA-3/ΔsdhA-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 31.5ºC 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 31.5ºC 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 ΔynfM-1/ΔynfM-2 primer pair, whereas the C-terminal fragment was amplified with the ΔynfM-3/ΔynfM-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 31.5ºC 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 31.5ºC. 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 31.5ºC for 16 h. The cells were then inoculated into 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·HCl)] and 50 g L^{-1} precipitated $MgCO_3$, after which they were sterilized by dry heat at 180ºC for 3 h (Nacalai Tesque, Kyoto, Japan). Following inoculation, succinate fermentation was performed at 31.5ºC 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, I employed a convenient evaluation system involving 1.5-mL microcentrifuge tubes (4,7). 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 31.5ºC 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 32ºC 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 the author's previous report (3). 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 (8). 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%, respectively (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 and 6.2 mM to undetected levels, respectively (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.

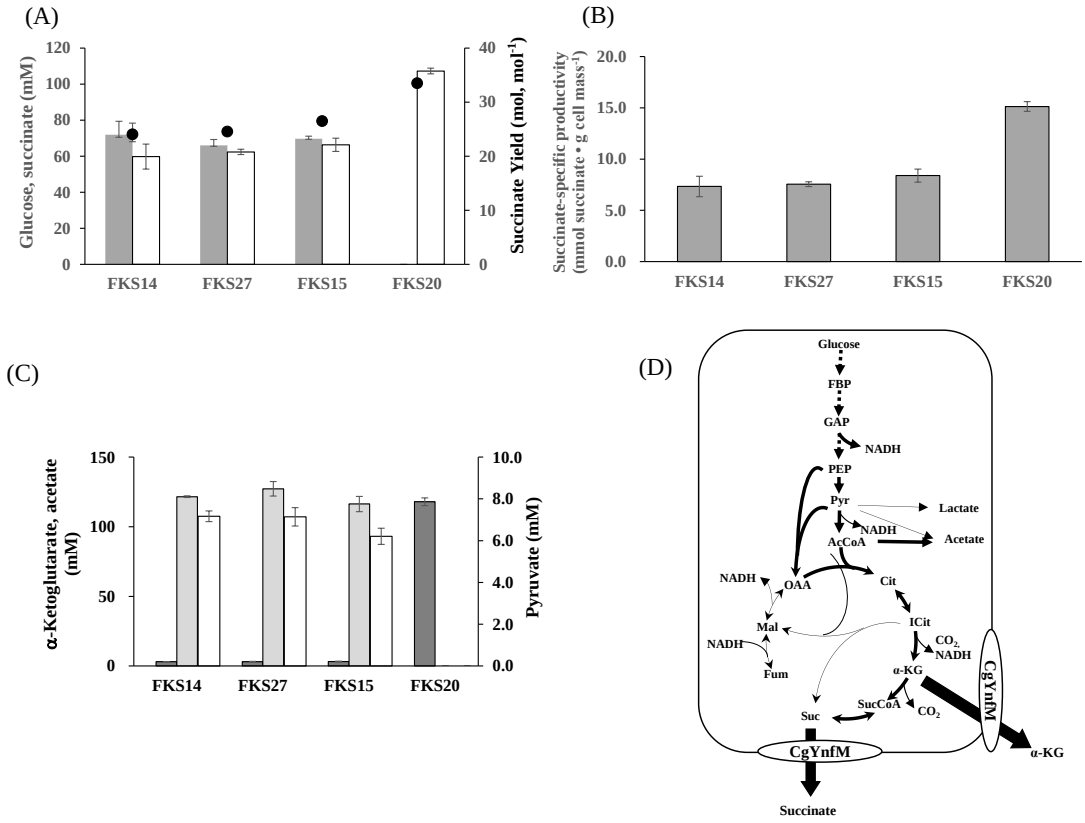

FIG. 1. Effect of *C. glutamicum CgynfM* expression on succinate production in *C. glutamicum* under aerobic conditions. Strain FKS14 was used as the host. *C. glutamicum* strains FKS14 (control), FKS27 (FKS14Δ*CgynfM*), FKS15 (FKS14/pVK9), and FKS20 (FKS14/pVK9::PmsrA-CgynfM) were used. Succinate production was performed in test tubes at 31.5ºC with a rotation speed of 120 rpm for 20 h under aerobic conditions. (A) The glucose concentration (gray columns), succinate concentration (white 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 gray columns), acetate concentration (light gray columns), and pyruvate concentration (white 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.

***CgynfM*-dependent succinate production in *C. glutamicum* lacking *sucE1*.** Previously, the author identified a novel succinate transporter, SucE1, in *C. glutamicum* (3). Furthermore, I 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 (3). Therefore, I 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.

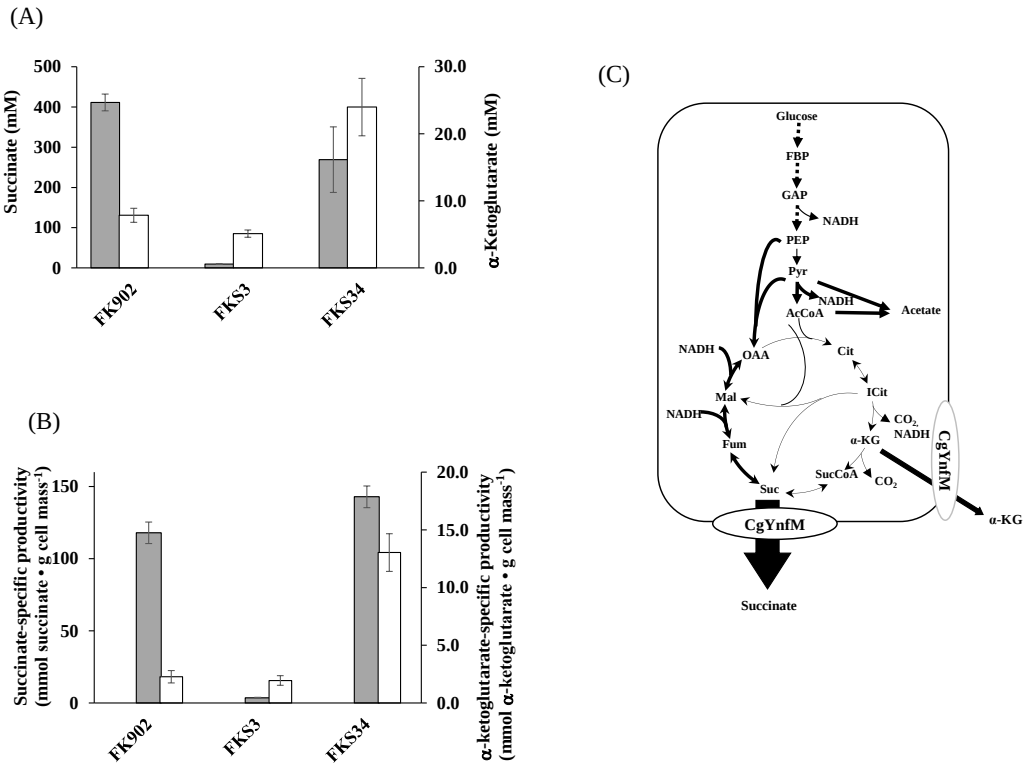

FIG. 2. *CgynfM*-dependent succinate production in the *sucE1*-deleted *C. glutamicum* strain. *C. glutamicum CgynfM* was introduced into *C. glutamicum* FK948, a strain in which the limiting step for succinate production is succinate export. The *C. glutamicum* FK902 (AJ110655/pVK9, control), FKS3 (AJ110655Δ*sucE1*/pVK9), and FKS34 (AJ110655Δ*sucE1*/pVK9::PmsrA-CgynfM) strains were used. Succinate production was performed in 1.5-mL microfuge tubes at 32ºC with a rotation speed of 1,400 rpm for 48 h under anaerobic conditions. (A) Succinate concentrations (gray columns) and αKG concentrations (white columns) are shown. (B) The succinate-specific productivity (mmol succinate • g cell mass^{-1}; gray columns) and αKG-specific productivity (mmol α-KG • g cell mass^{-1}; white 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.

Comparison of succinate transporters by succinate production in the *sucE1* deletion *strain.* The author previously found that not only SucE1, but also YjjPB from *E. aerogenes*, functions as a dicarboxylate transporter (9). Next, the substrate specificities of CgYnfM were compared to those of AeYjjPB and SucE1 by determining succinate production in the *sucE1*-deletion strain. The author 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.

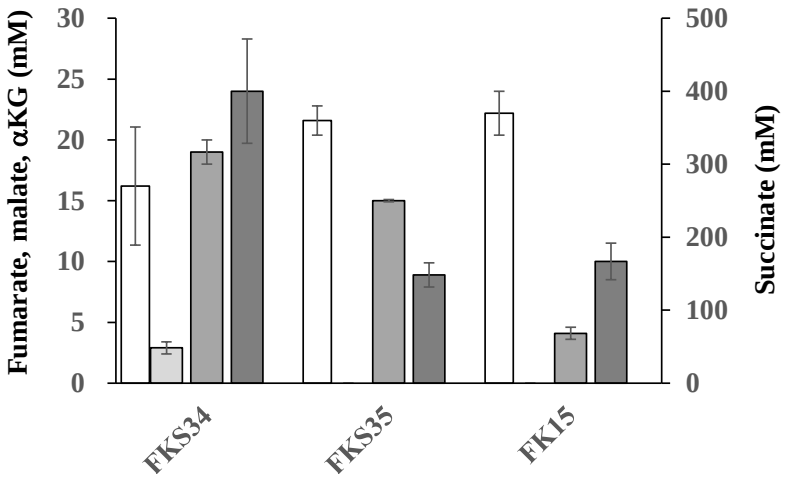

FIG. 3. Comparison of succinate-production levels after expressing different succinate transporters in the *sucE1*-deletion strain. *C. glutamicum CgynfM*, *sucE1*, and *E. aerogenes AeyjjPB* were introduced into *C. glutamicum* FK948, a strain in which the limiting step for succinate production is succinate export. Succinate production was performed in 1.5-mL microfuge tubes at 32ºC with a rotation speed of 1,400 rpm for 48 h under anaerobic conditions. (A) The concentrations of succinate (white 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.

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* (10). To overcome this issue, I 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, I 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 (4,9). The assay indicated that CgYnfM partially restored succinate production (Fig. 2A), suggesting that CgYnfM could function as a succinate transporter. In this study, I demonstrated that expression of succinate transporters, not only SucE1 (3), YjjPB (9), and EaYjjPB (4), but also CgYnfM, has a large impact on aerobic succinate production in *C. glutamicum*. In the future, I 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 (11), which converts αKG into succinyl-CoA.

When performing the *in vivo* transport assays (Fig. 2), I 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 (3). 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 (12), 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 (13). 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 (14). In the sequence alignment of *C. glutamicum* KGTPs and *C. glutamicum* CgYnfM (Fig. 4B), I 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.

After expressing known succinate transporters in the *sucE1*-deletion strain, I 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 (15-17). Malic acid is also useful for producing pharmaceuticals, cosmetics, and acidulants in the food industry (18, 19). CgYnfM may be effective for producing dicarboxylates, including αKG.

In conclusion, I 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 my 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.

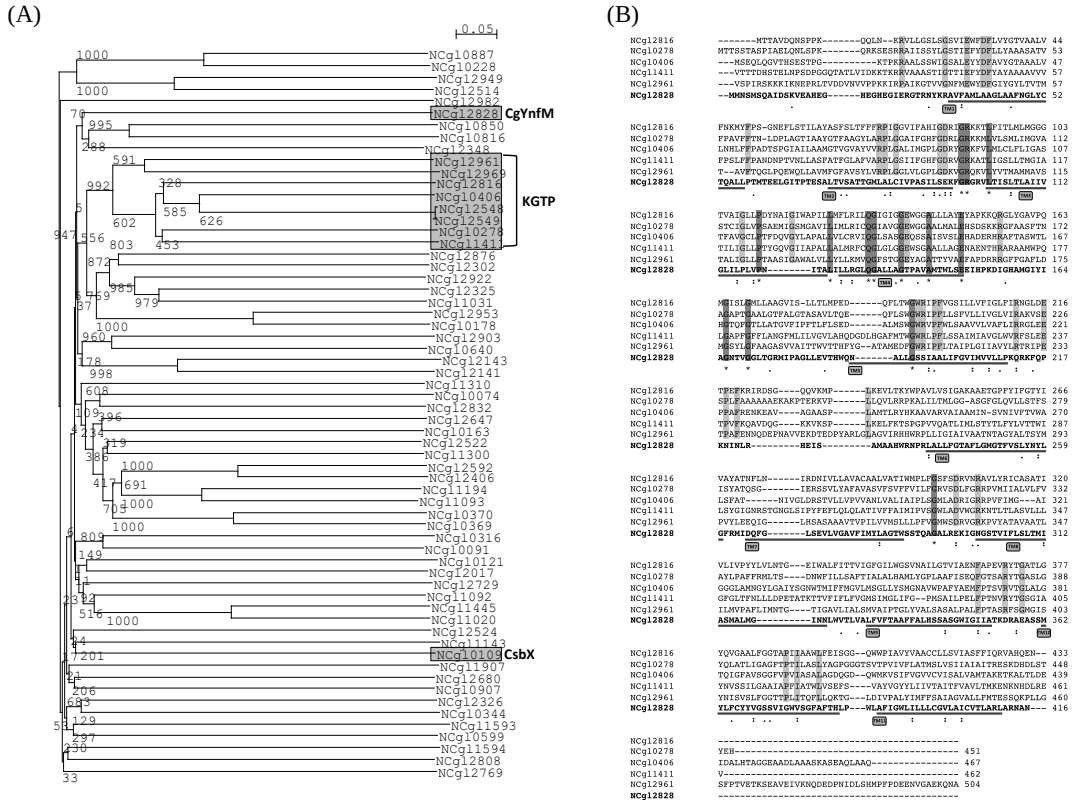

FIG. 4. Phylogenic analysis of amino acid sequences of *C. glutamicum* MFS transporters (A) and multiple-sequence alignment of KGTP homologs and CgynfM (B). (A) The amino acid sequences of *C. glutamicum* MFS transporters were aligned using ClustalW (2.1) (DNA Data Bank of Japan), and the phylogenetic 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).

REFERENCES

1. **Sambrook, J. and Russell, D.W.:** Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor, New York (2001).
2. **Nakamura, J., Hirano, S., Ito, H., and Wach, M.:** Mutations of the *Corynebacterium glutamicum* NCgl1221 gene, encoding a mechanosensitive channel homolog, induce L-glutamic acid production, Appl. Environ. Microbiol., **73**, 4491–4498 (2007).
3. **Fukui, K., Koseki, C., Yamamoto, Y., Nakamura, J., Sasahara, A., Yuji, R., Hashiguchi, K., Usuda, Y., Matsui, K., Kojima, H., and Abe, K.:** Identification of succinate exporter in *Corynebacterium glutamicum* and its physiological roles under anaerobic conditions, J Biotechnol., **154**, 25–34 (2011).
4. **Fukui, K., Nanatani, K., Hara, Y., Yamakami, S., Tokura, M., and Abe, K.:** Identification of *EayjjPB* encoding a dicarboxylate transporter important for succinate production under aerobic and anaerobic conditions in *Enterobacter aerogenes*, J. Biosci. Bioeng., **125**, 505–512 (2018).
5. **Chinen, A., Kozlov, Y.I., Hara, Y., Izui, H., and Yasueda, H.:** Innovative metabolic pathway design for efficient L-glutamate production by suppressing CO_2 emission, J. Biosci. Bioeng., **103**, 262–269 (2007).
6. **Miwa, K., Matsui, K., Terabe, M., Ito, K., Ishida, M., Takagi, H., Nakamori, S., and Sano, K.:** Construction of novel shuttle vectors and a cosmid vector for the glutamic acid-producing bacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*, Gene, **39**, 281–286 (1985).
7. **Tajima, Y., Yamamoto, Y., Fukui, K., Nishio, Y., Hashiguchi, K., Usuda, Y., and**

Sode, K.: Effects of eliminating pyruvate node pathways, and co-expression of heterogeneous carboxylation enzymes, on succinate production by *Enterobacter aerogenes*, Appl. Environ. Microbiol., **81**, 929–937 (2015).

8. **Litsanov, B., Kabus, A., Brocker, M., and Bott, M.:** Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*, Microb. Biotechnol., **5**, 116–128 (2012).
9. **Fukui, K., Nanatani, K., Hara, Y., Yamakami, S., Yahagi, D., Chinen, A., Tokura, M., and Abe, K.:** *Escherichia coli yjjPB* genes encode a succinate transporter important for succinate production, Biosci. Biotechnol. Biochem., **4**, 1–8 (2017).
10. **Zhu, N., Xia, H., Wang, Z., Zhao, X., and Chen, T.:** Engineering of acetate recycling and citrate synthase to improve aerobic succinate production in *Corynebacterium glutamicum*, PLoS ONE, **8**, e60659 (2013).
11. **Usuda, Y., Tujimoto, N., Abe, C., Asakura, Y., Kimura, E., Kawahara, Y., Kurahashi, O., and Matsui, H.:** Molecular cloning of the *Corynebacterium glutamicum* ('*Brevibacterium lactofermentum*' AJ12036) *odhA* gene encoding a novel type of 2-oxoglutarate dehydrogenase., Microbiology, **142**, 3347–3354 (1996).
12. **Saier, M.H., Reddy, V.S., Tsu, B.V., Ahmed, M.S., Li, C., and Moreno-Hagelsieb, G.:** The Transporter Classification Database (TCDB): recent advances. Nucleic Acids Res., **44**, D372–D379 (2016).
13. **Seol, W. and Shatkin, A.J.:** *Escherichia coli* α-ketoglutarate permease is a constitutively expressed proton symporter, J. Biol. Chem., **267**, 6409–6413 (1992).
14. **Koita, K. and Rao, C.V.:** Identification and analysis of the putative pentose sugar

efflux transporters in *Escherichia coli*, PLoS One, **7**, e43700 (2012).

15. **Chernyavskaya, O.G., Shishkanova, N.V., Il'chenko, A.P., and Finogenova, T.V.:** Synthesis of alpha-ketoglutaric acid by *Yarrowia lipolytica* yeast grown on ethanol, Appl. Microbiol. Biotechnol., **53**, 152–158 (2000).
16. **Huang, H.J., Liu, L.M., Li, Y., Du, G.C., and Chen, J.:** Redirecting carbon flux in Torulopsis glabrata from pyruvate to alpha-ketoglutaric acid by changing metabolic co-factors, Biotechnol. Lett., **28**, 95–98 (2006).
17. **Stottmeister, U., Aurich, A., Wilde, H., Andersch, J., Schmidt, S., and Sicker, D.:** White biotechnology for green chemistry: fermentative 2-oxocarboxylic acids as novel building blocks for subsequent chemical syntheses, J. Ind. Microbiol. Biotechnol., **32**, 651–664 (2005).
18. **Bressler, E., Pines, O., Goldberg, I., and Braun, S.:** Conversion of fumaric acid to L-malic by sol-gel immobilized *Saccharomyces cerevisiae* in a supported liquid membrane bioreactor., Biotechnol. Prog. **18**, 445–450 (2002).
19. **Rosenberg, M., Mikova, H. and Kristofikova, L.:** Formation of L-malic acid by yeast of the genus *Dipodascus*. Lett. Appl. Microbiol., **29**, 221–223 (1999).

Summary

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, the author identified *ynfM* in *Pantoea ananatis* (*PaynfM)* as a gene encoding a dicarboxylate transporter and found a homologous gene (*CgynfM*) in *Corynebacterium glutamicum*. In this study, the author 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 from 120 mM and 6.2 mM, respectively, to undetectable levels. CgYnfM restored succinate production under anaerobic conditions in *C. glutamicum* strain AJ110655Δ*sucE*1, 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.

CONCLUSION

As I said in the General Introduction, organic acids have long been used as raw materials in the food, chemical, and pharmaceutical industries. Moreover, there is a growing market for lactate and succinate polymers as alternative raw materials for biodegradable plastics. Succinate is produced via the reductive or oxidative arm of the tricarboxylic acid cycle pathway in various bacteria, such as *Corynebacterium glutamicum, Escherichia coli, Enterbacter aerogenes.* On the other hand, it is known that exporters are important for microbial production. Therefore, I studied some screens to identify succinate transporters in *C. glutamicum*, *E.coli*, and *E. aerogenes*, and examined the effect of them on microbial succinate production under aerobic and anaerobic condition.

In Chapter I, I identified *sucE1* of *C. glutamicum* as a novel succinate exporter gene by transcriptome analysis. *sucE1* expression levels were higher under microaerobic conditions than aerobic conditions, and that overexpression or disruption of *sucE1* respectively increased or decreased succinate productivity during fermentation. Homology and transmembrane helix searches identified SucE1 as a membrane protein belonging to the aspartate:alanine exchanger (AAE) family. Partially purified histidine-tagged SucE1 reconstituted in succinate-loaded liposomes clearly demonstrated transport activities for succinate. These findings suggest that *sucE1* encodes a novel succinate exporter that is induced under microaerobic conditions, and is important for succinate production.

In Chapter II, I identified the *yjjPB* genes encoding a succinate transporter by screening an *E. coli* MG1655 genome library. Then, I found that they belong to a split

type of ThrE family and are important for succinate production. A complementation assay conducted in *C. glutamicum* strain AJ110655Δ*sucE*1 demonstrated that both YjjP and YjjB are required for the restoration of succinate production, which suggests that YjjPB constitutes a succinate transporter in *E. coli* and that the products of both genes are required for succinate export. On the other hand, I found that the *E. aerogenes*, which is a same gram-negative bacterium as *E.coli*, has the homologs (*EayjjPB*). Then I demonstrated that they are dicarboxylate transporter important for succinate production, and the products of both genes are also required for dicarboxylate transport.

In Chapter III, I found a homologous gene of the *ynfM* in *C. glutamicum*, which is known as a dicarboxylate transporter in *Pantoea ananatis*. As the result of analysis of the homolog (*CgynfM*), I demonstrated that CgYnfM from *C. glutamicum* functions as a dicarboxylate transporter that is applicable to not only succinate production, but also other dicarboxylate such as α-ketoglutarate.

In conclusion, I discovered succinate transporters in various bacteria, and demonstrated that they are significantly important for succinate production.

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LIST OF PUBLICATIONS

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Fukui K, Nanatani K, Hara Y, Yamakami S, Yahagi D, Chinen A, Tokura M, Abe K.: *Escherichia coli yjjPB* genes encode a succinate transporter important for succinate production. *Biosci Biotechnol Biochem.*, 81 (9), 1837-1844 (2017)

Fukui K, Nanatani K, Hara Y, Tokura M, Abe K.: Identification of *EayjjPB* encoding a dicarboxylate transporter important for succinate production under aerobic and anaerobic conditions in *Enterobacter aerogenes*. *J Biosci Bioeng.*: 125 (5), 505-512 (2018)

Fukui K, Nanatani K, Nakayama M, Hara Y, Tokura M, Abe K.: *Corynebacterium glutamicum CgynfM* encodes a dicarboxylate transporter applicable to succinate production. *J Biosci Bioeng.* (2018)