APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Activating C$_4$-dicarboxylate transporters DcuB and DcuC for improving succinate production

Jing Chen · Xinna Zhu · Zaigao Tan · Hongtao Xu · Jinlei Tang · Dongguang Xiao · Xueli Zhang

Abstract Although many efforts had been performed to engineer *Escherichia coli* for succinate production, succinate efflux system had not been investigated as an engineering target for improving succinate production. In this work, four Dcu transporters, which had been reported to be responsible for C$_4$-dicarboxylates transportation of *E. coli*, were investigated for their succinate efflux capabilities. These four *dcu* genes were deleted individually in a previously constructed succinate-producing strain to study their effects on succinate production. Deleting *dcuA* and *dcuD* genes had nearly no influence, while deleting *dcuB* and *dcuC* genes led to 15 and 11 % decrease of succinate titer, respectively. Deleting both *dcuB* and *dcuC* genes resulted in 90 % decrease of succinate titer, suggesting that DcuB and DcuC were the main transporters for succinate efflux and they functioned as independent and mutually redundant succinate efflux transporters. Furthermore, RBS library having strengths varied from 0.17 to 8.6 times of induced *E. coli lacZ* promoter was used to modulate *dcuB* and *dcuC* genes for improving succinate production. Modulating these two genes in combination led to 34 % increase of succinate titer. To the best of knowledge, this was the first report about improving succinate production through engineering succinate efflux system.

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Introduction

Succinate is a four-carbon dicarboxylate that has many applications in food, agricultural, pharmaceutical, and biodegradable plastic industries (McKinlay et al. 2007). Although succinate is currently produced from petroleum-derived maleic anhydride, considerable interest in microbial production from renewable biomass has emerged during the past two decades (Lee et al. 2005; Lin et al. 2005; Wang et al. 2006; McKinlay et al. 2007; Zhang et al. 2009a). Many efforts had been performed to improve succinate production in *Escherichia coli*. Competitive fermentation pathways, such as formate pyruvate-lyase, lactate dehydrogenase, and alcohol dehydrogenase, were usually inactivated to eliminate production of byproducts and increase succinate yield (Stols and Donnelly 1997; Lee et al. 2005; Wang et al. 2006; Jantama et al. 2008a, b; Singh et al. 2011). In addition, precursor and energy supplies were increased for improved cell growth and succinate production under anaerobic conditions (Chatterjee et al. 2001; Zhang et al. 2009a, b; Singh et al. 2011).

On the other hand, transportations, such as substrate uptake and product efflux, are very important for efficient production of target compounds. The phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS) of *E. coli* was usually inactivated to increase PEP supply for succinate production, and alternative glucose utilization pathways were recruited to improve cell growth and glucose consumption rates (Wang et al. 2006; Lu et al. 2012; Tang et al. 2013). However, there has been no report about engineering export system to improve succinate production.

E. coli contains four Dcu carries (encoded by *dcuA*, *dcuB*, *dcuC*, and *dcuD* genes) for C$_4$-dicarboxylates (succinate,

fumarate, and malate) transportation. The uptake, antiport, and efflux of C_4-dicarboxylates are mediated by the Dcu transporters. DcuA and DcuB are homologous proteins that appear to function as independent and mutually redundant C_4-dicarboxylate transporters during anaerobic conditions (Golby et al. 1998). DcuB is the major C_4-dicarboxylate carrier for fumarate respiration with high fumarate-succinate exchange activity, while the precise physiological purpose of DcuA remains unclear (Golby et al. 1998). Although DcuC is able to compensate for DcuA and DcuB in fumarate-succinate exchange and fumarate uptake, the preferential function of DcuC is suggested to be involved in succinate efflux during glucose fermentation (Zientz et al. 1999). DcuD, which is homologous to DcuC, apparently is not expressed under most conditions, and its role is unknown (Janausch and Unden 1999).

In this work, the four *dcu* genes were individually deleted in a succinate-producing strain Suc-T110 (Tan et al. 2013) to investigate their roles in succinate production under anaerobic glucose fermentation conditions. Deleting *dcuA* and *dcuD* genes had nearly no influence, while deleting *dcuB* and *dcuC* genes led to 15 and 11 % decrease of succinate titer, respectively. Deleting both *dcuB* and *dcuC* genes resulted in 90 % decrease of succinate titer. It was suggested that DcuB and DcuC were the main transporters for succinate efflux during glucose fermentation. These two genes were further modulated in combination by ribosomal binding site (RBS) libraries, leading to 34 % increase of succinate titer.

Materials and methods

Strains, medium, and growth conditions

Strains used in this study were listed in Table 1. During strain construction, cultures were grown aerobically at 30, 37, or 39 °C in Luria broth (per liter, 10 g Difco tryptone, 5 g Difco yeast extract, and 10 g NaCl). Ampicillin (100 mg L^{-1}), kanamycin (25 mg L^{-1}), and chloramphenicol (17 mg L^{-1}) were used where appropriate.

Deletion of *dcu* genes in strain Suc-T110

The *dcuA* gene in strain Suc-T110 (Tan et al. 2013) was deleted using a two-step recombination method as described previously (Zhang et al. 2007; Jantama et al. 2008a). In the first recombination, *cat-sacB* cassette was amplified from pXZ-CS (Tan et al. 2013) using primer set dcuA-QC-cat-up/dcuA-QC-sacB-down and used to replace the coding region of *dcuA* gene. Fusion PCR was performed to prepare DNA fragment for the second recombination. The upstream and downstream regions (about 700 bp) of *dcuA* gene were amplified from *E. coli* ATCC 8739 genomic DNA using primer sets dcuA-QC-1/dcuA-QC-2 and dcuA-QC-

Table 1 Strains and plasmids used in this study

Name	Characteristics	Source or reference
Strains		
ATCC 8739	Wild type	Lab collection
M1-93	ATCC 8739, FRT-*Km*-FRT-M1-93-*lacZ*	Lu et al. 2012
Suc-T110	ATCC8739, Δ*ldhA*, Δ*pflB*, Δ*ptsI*, P*pck**-*galP*, P*pck**-*pck*	Tan et al. 2013
JC-101	Suc-T110, Δ*dcuA*	This study
JC-102	Suc-T110, Δ*dcuB*	This study
JC-103	Suc-T110, Δ*dcuC*	This study
JC-104	Suc-T110, Δ*dcuD*	This study
JC-105	Suc-T110, Δ*dcuB*, Δ*dcuC*	This study
Suc-T110-ducB1	Suc-T110, RBSL1-*dcuB*	This study
Suc-T110-ducB2	Suc-T110, RBSL2-*dcuB*	This study
JC-205	Suc-T110, RBSL3-*dcuB*	This study
JC-206	Suc-T110, RBSL4-*dcuB*	This study
Suc-T110-ducB5	Suc-T110, RBSL5-*dcuB*	This study
Suc-T110-ducC1	Suc-T110, RBSL1-*dcuC*	This study
JC-207	Suc-T110, RBSL2-*dcuC*	This study
JC-208	Suc-T110, RBSL3-*dcuC*	This study
Suc-T110-ducC4	Suc-T110, RBSL4-*dcuC*	This study
Suc-T110-ducC5	Suc-T110, RBSL5-*dcuC*	This study
JC-209	Suc-T110, RBSL3-*dcuB*, RBSL2-*dcuC*	This study
JC-210	Suc-T110, RBSL3-*dcuB*, RBSL3-*dcuC*	This study
JC-211	Suc-T110, RBSL4-*dcuB*, RBSL2-*dcuC*	This study
JC-212	Suc-T110, RBSL4-*dcuB*, RBSL3-*dcuC*	This study
Plamids		
pXZ-CS	*cat* gene of pACYC184 and *sacB* gene from *Bacillus subtilis* cloned into pEASY-Blunt vector	Tan et al. 2013

pck* represents a mutated form of *pck* (G to A at -64 relative to the ATG start) (Zhang et al. 2009a)

3/dcuA-QC-4. These two fragments were then served as templates to perform fusion PCR using primer set dcuA-QC-1/dcuA-QC-4. The resulting DNA fragment (about 1,400 bp) was used to replace *cat-sacB* cassette by selection for resistance to sucrose. Cells containing *sacB* gene accumulated levan during incubation with sucrose and were killed. Surviving recombinants were highly enriched for loss of *cat-sacB* cassette (Zhang et al. 2007; Jantama et al. 2008a). The *dcuB*, *dcuC*, and *dcuD* genes were deleted in the same manner. All strains obtained were listed in Table 1, and all primers were listed in Table 2.

Modulating *lacZ* gene with RBS library

A one-step homologous recombination method was used to modulate *lacZ* gene with RBS library (Fig. 1). DNA fragment

Table 2 Primers used in this study

dcuA gene deletion	
dcuA-QC-1	CTCTATCGGTATCGTTA
dcuA-QC-2	ATTAGCCTTCCTTGTTTTTTAAC
dcuA-QC-3	GGAAGGCTAATTGATCAATCGCGGGG
dcuA-QC-4	CAAAGTAGCAGGATCAGCGT
dcuA-QC-cat-up	GAGCAGTAACTTAAAAATAACAATCTAATATCAACTTGTTAAAAAACAAGGAAGGCTAATTGTGACGGAAGATCACTTCGCA
dcuA-QC-sacB-down	GGGGGAAGGATGTTAGTCGGCGGGAAAGAAAGCGGGGCGTGAACGCCCCGCGATTGATCATTATTTGTTAACTGTTAATTGTCCT
dcuB gene deletion	
dcuB-QC-1	CCTCATCTGGCTGGTCAA
dcuB-QC-2	GTGTGAACCCTCGCGATAA
dcuB-QC-3	GGGTTCACACTGCACTTTGCGTGCCG
dcuB-QC-4	TGCCGCCAATCACAAACG
dcuB-QC-cat-up	CGTGACTGTGATCTATTCAGCAAAAATTTAAATAGGATTATCGCGAGGGTTCACACTGTGACGGAAGATCACTTCGCA
dcuB-QC-sacB-down	ATTTGTTATTCGAAAATGGCGTGCCGCGTAGTCACGGGCGGCACGCAAAGTGCATTATTTGTTAACTGTTAATTGTCCT
dcuC gene deletion	
dcuC-QC-1	TTTTCTGCGATGGGAATAGT
dcuC-QC-2	TTTTCTGCGATGGGAATAGT
dcuC-QC-3	CAGGAAAAATTATAGTAATGCCGGAGGCA
dcuC-QC-4	CGGTGTCTGAAGCCGTAC
dcuC-QC-cat-up	CCTTAAGCTTTAGGAATTTTTTATTATTTACTTTGGGGCCTGGAGACAGGAAAAATTTGTGACGGAAGATCACTTCGCA
dcuC-QC-sacB-down	GCATATACCACAGTGGGTGACATGATAAAGCCGGAGGAAACTTGCCTCCGGCATTACTATTTATTTGTTAACTGTTAATTGTCCT
dcuD gene deletion	
dcuD-QC-1	GTTCACCTTCGCCAAAT
dcuD-QC-2	TGTTGAACTCCGTGTCAAAAGAAAACGGTCAATCC
dcuD-QC-3	ACACGGAGTTCAACAGCCATTAAAAGACAAAACAG
dcuD-QC-4	GCGTGACGGGCAAATC
dcuD-QC-cat-up	TCGTTGTTTTCAATCTGCCGTTTATGGGATTGACCGTTTTCTTTTGACACGGAGTTCAACATGTGACGGAAGATCACTTCGCA
dcuD-QC-sacB-down	ATTACGCGTTGTGAAGTAATACAAAAACAGGCCCTGGTGGCCTGTTTTGTCTTTTAATGGCTTATTTGTTAACTGTTAATTGTCCT
Modulation of *lacZ* gene	
lacI-up500	CGGGCGACGTTTGCCGCTTCTGAAAACC
lacZ-RBSL-down	CAGTCACGACGTTGTAAAACGACGGCCAGTGAATCCGTAATCATGGTCATNNNNNNYCTCCTGGTTTAAACGTACATG
lacZ-373	AGTAACAACTCGTCGGATTCT
Modulation of *dcuB* gene	
dcuB-cat-up-1	CAGAACGTGACTGTGATCTATTCAGCAAAAATTTAAATAGGATTATCGCGAGGGTTCACACTGTGACGGAAGATCACTTCGCA
dcuB-sacB-down	CCCCAATCTGGCGCCCAAGAAGATCGCCAGCAAAACTATGATGAGTTCTACAACTAGCATTTATTTGTTAACTGTTAATTGTCCT
dcuB-P-up-1	CAGAACGTGACTGTGATCTATTCAGCAAAAATTTAAATAGGATTATCGCGAGGGTTCACACTTATCTCTGGCGGTGTTGAC
dcuB-RBS-down	CTTTCGGGCACCATAAAACAGACATATCAGTATTATGATAAGTTGGATAGTAAATAACAT AGCTGTTTCCTG
dcuB-RBSL-down	GGCACCATAAAACAGACATATCAGTATTATGATAAGTTGGATAGTAAATAACATNNNNNNYCTCCTGGTTTAAACGTACATG
dcuB-down-200	ACCACCGCAATGATAACCAG

Table 2 (continued)

Modulation of *dcuC* gene	
dcuC-cat-up	CAGAACGTGACTGTGATCTATTCAGCAAAAATTTAAATAGGATTATCGCGAGGGTTCACACTGTGACGGA AGATCACTTCGCA
dcuC-sacB-down	CCCCAATCTGGCGCCCAAGAAGATCGCCAGCAAAACTATGATGAGTTCTACAACTAGCATTTATTTGTTA ACTGTTAATTGTCCT
dcuC-P-up	CAGAACGTGACTGTGATCTATTCAGCAAAAATTTAAATAGGATTATCGCGAGGGTTCACACTTATCTCTG GCGGTGTTGAC
dcuC-RBS-down	CTTTCGGGCACCATAAAACAGACATATCAGTATTATGATAAGTTGGATAGTAAATAACAT AGCTGTTTCCTG
dcuC-RBSL-down	ACACCCACAATAACCACAACCCCAATAAGGAGTTCAATGAATGTCAGCATNNNNNNYCTCCTGGTTTAAA CGTACATG
dcuC-down-200	ACCACCGCAATGATAACCAG

containing RBS library was amplified from genomic DNA of recombinant *E. coli* M1-93 using primer set lacI-up500/lacZ-RBSL-down and used for recombination. Recombinant *E. coli* M1-93 was selected from a previously constructed mRNA stabilizing region library, which had a regulatory part inserted in front of *lacZ* gene, and its strength was five times of induced *E. coli lacZ* promoter (Lu et al. 2012). The left homologous arm was 500 bp. The partial coding region of *lacZ* gene (from +1 to +50 relative to the translational start site of *lacZ*) of *E. coli* was used as the right homologous arm (Fig. 1b). The sequence of RBS library is CAGGAGRNNN NNN (Fig. 1a). After recombination, colonies were randomly picked for further PCR verification using primer set lacI-up500/lacZ-373. Twenty-nine correct colonies and wild-type strain ATCC 8739 were then picked and grown in LB medium for β-galactosidase assay.

Enzyme assays

Exponentially grown cells were harvested by centrifugation (7,000 g for 5 min, 4 °C). Cells were washed twice in 50 mM sodium phosphate buffer (pH 7.0), and the activity of β-galactosidase was measured as described by Miller (1992).

Modulating *dcuB* and *dcuC* genes with RBS library

A two-step homologous recombination method was used to modulate *dcuB* gene with RBS library. Firstly, *cat-sac*B cassette was amplified from pXZ-CS with primer set dcuB-cat-up-1/dcuB-sacB-down and used to insert before ATG translation start site of *dcuB* gene. DNA fragment containing RBS library, which was the same as the RBS library of *lacZ* gene, was amplified from genomic DNA of recombinant *E. coli* M1-93 using primer set dcuB-up-P-1/dcuB-RBSL-down and used for the second recombination. Colonies without chloramphenicol resistant were picked for further PCR verification using primer set Ap1-up/dcuB-down-200. Five right colonies were selected for further fermentation. The *dcuC* gene was modulated in a same manner.
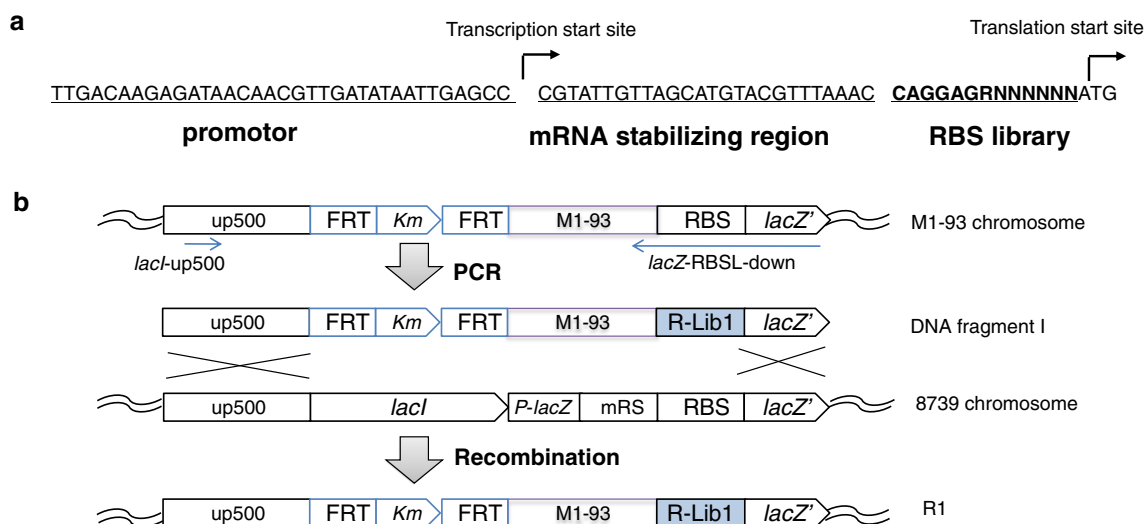
Fig. 1 Construction of RBS library in front of *E. coli lacZ* gene with one-step recombination method. **a** Structure and sequence of RBS library. **b** Method for constructing RBS library in front of *E. coli lacZ* gene

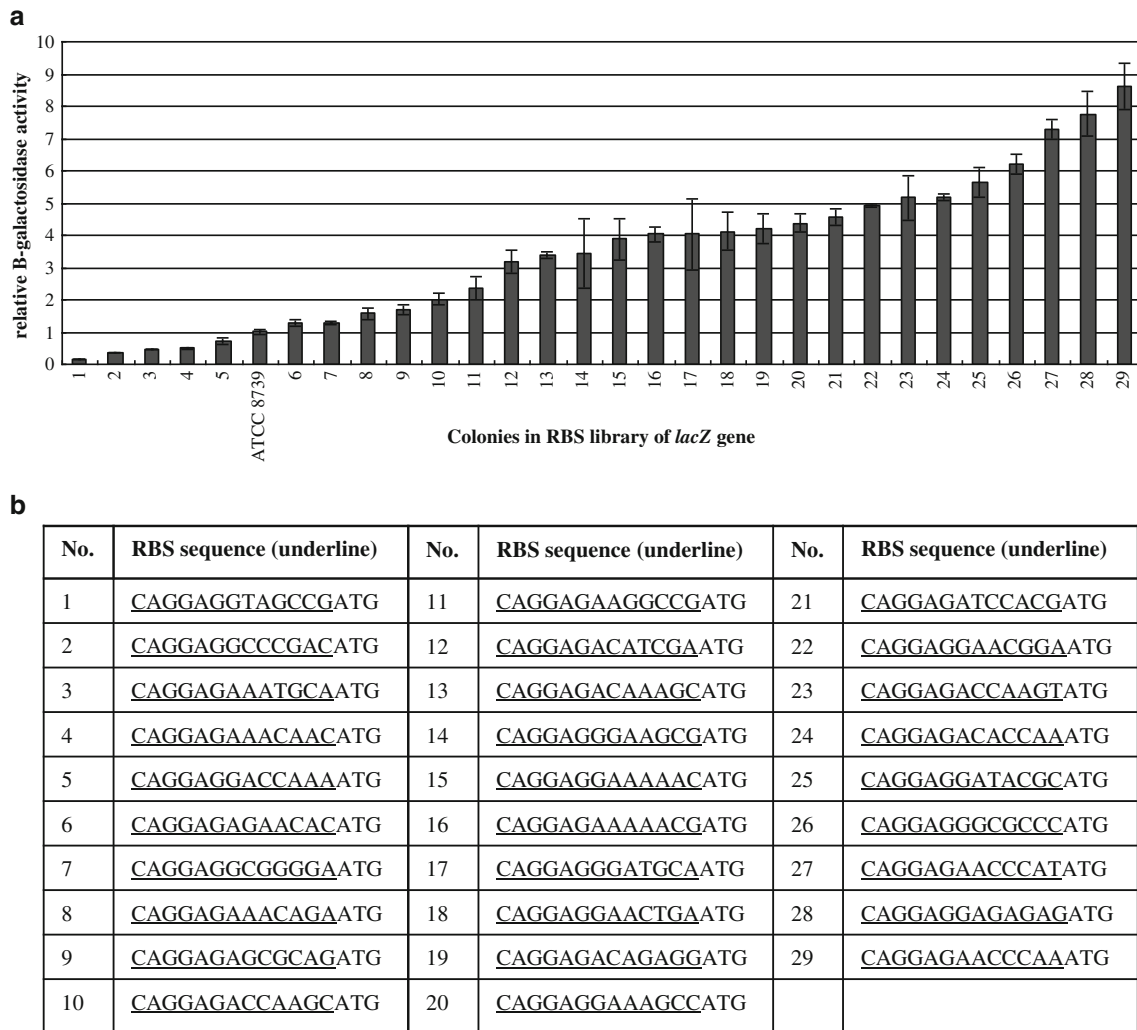**Table 3** Effects of deleting *dcu* genes on succinate production

Strain[a]	Characteristics	Cell mass (g/L)	Glu used (mM)	Suc yield (mol/mol)	Fermentation products (mM)		
					Suc	Ace	Eth
Suc-T110		1.53	244±5	1.12±0.01	273±5	96±4	4±2
JC-101	Suc-T110, Δ*dcuA*	1.61	236±3	1.12±0.02	267±5	71±6	10±2
JC-102	Suc-T110, Δ*dcuB*	1.55	245±5	0.95±0.01	232±6	57±4	30±4
JC-103	Suc-T110, Δ*dcuC*	1.66	243±4	1.01±0.02	244±6	55±5	32±3
JC-104	Suc-T110, Δ*dcuD*	1.55	238±3	1.13±0.02	268±5	73±4	11±2
JC-105	Suc-T110, Δ*dcuB*, Δ*dcuC*	0.60	63±3	0.41±0.01	26±3	30±5	56±6

[a] Fermentations were carried out in NBS mineral salts medium containing 5 % glucose and 100 mM potassium bicarbonate for 72 h. The pH was maintained at 7.0 by automatic addition of a base containing 2.4 M potassium carbonate and 1.2 M potassium hydroxide

Fermentation

Fresh colonies were picked from New Brunswick Scientific (NBS) mineral salts plates containing 20 g L^{-1} glucose, and inoculated into 250 ml flasks containing 100 ml NBS mineral salts medium with 50 g L^{-1} glucose, and grown at 37 °C and 100 rpm for 12 h. Cultures were then transferred to a 500 ml fermentation vessel containing 250 ml NBS

No.	RBS sequence (underline)	No.	RBS sequence (underline)	No.	RBS sequence (underline)
1	CAGGAGGTAGCCGATG	11	CAGGAGAAGGCCGATG	21	CAGGAGATCCACGATG
2	CAGGAGGCCCGACATG	12	CAGGAGACATCGAATG	22	CAGGAGGAACGGAATG
3	CAGGAGAAATGCAATG	13	CAGGAGACAAAGCATG	23	CAGGAGACCAAGTATG
4	CAGGAGAAACAACATG	14	CAGGAGGGAAGCGATG	24	CAGGAGACACCAAATG
5	CAGGAGGACCAAAATG	15	CAGGAGGAAAAACATG	25	CAGGAGGATACGCATG
6	CAGGAGAGAACACATG	16	CAGGAGAAAAACGATG	26	CAGGAGGGCGCCCATG
7	CAGGAGGCGGGGAATG	17	CAGGAGGGATGCAATG	27	CAGGAGAACCCATATG
8	CAGGAGAAACAGAATG	18	CAGGAGGAACTGAATG	28	CAGGAGGAGAGAGATG
9	CAGGAGAGCGCAGATG	19	CAGGAGACAGAGGATG	29	CAGGAGAACCCAAATG
10	CAGGAGACCAAGCATG	20	CAGGAGGAAAGCCATG		

Fig. 2 Expression strengths and RBS sequences of colonies in the RBS library. **a** Relative expression strengths of 29 colonies in the RBS library. The strength was depicted as the relative strength to induced native *E. coli lacZ* promoter. **b** RBS sequences of 29 colonies in the RBS library
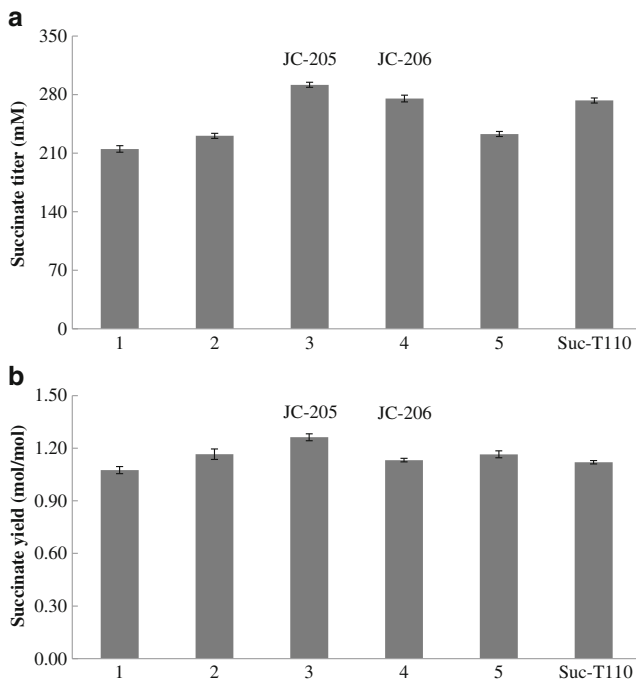
Fig. 3 Production of succinate by five colonies from the RBS library of *dcuB* gene. **a** succinate titer; **b** succinate yield

mineral salts medium with 50 g L^{-1} glucose and 100 mM potassium bicarbonate. The initial OD_{550} was 0.1, and pH was maintained at 7.0 by automatic addition of a base containing 2.4 M potassium carbonate and 1.2 M potassium hydroxide.

Analysis

The dry weight of cells was calculated by measuring the optical density value at 550 nm (OD550). Organic acids and

residual glucose in fermentation broth were measured by high-performance liquid chromatography (Zhang et al. 2009b).

Results

Effects of deleting *dcu* genes on succinate production

In order to investigate the roles of different Dcu transporters on succinate efflux during glucose fermentation, the four *dcu* genes of strain Suc-T110 were deleted individually. This parent strain was previously engineered for succinate production by deleting lactate dehydrogenase and pyruvate formate–lyase genes to inactivate competitive fermentation pathways, as well as inactivating PTS system and recruiting an alternative glucose utilization pathway (Galactose permease) to increase PEP supply. Furthermore, PEP carboxykinase was activated to increase energy supply (Tan et al. 2013). This strain produced 273 and 280 mM succinate after fermentation for 72 and 96 h, respectively (Table 3). Succinate yield of this strain was 1.12 mol/mol (Table 3).

Deleting *dcuA* or *dcuD* gene had nearly no influence on succinate titer and yield (Table 3). Succinate titer decreased 15 % (from 273 to 232 mM) and succinate yield decreased 15 % (from 1.12 to 0.95 mol/mol) after deleting *dcuB* gene. Succinate titer decreased 11 % (from 273 to 244 mM) and succinate yield decreased 10 % (from 1.12 to 1.01 mol/mol) after deleting *dcuC* gene. The *dcuB* and *dcuC* genes were further deleted simultaneously, resulting in strain JC-105. Cell mass, succinate titer, and succinate yield of strain JC-105 decreased significantly. This strain produced only 26 mM succinate with a yield of 0.41 mol/mol. Succinate titer and

Table 4 Effects of modulating *dcuB* and *dcuC* genes on succinate production

Strain[a]	Characteristics[b]	Cell mass (g/L)	Glu used (mM)	Suc yield (mol/mol)	Fermentation products (mM)		
					Suc	Ace	Eth
Suc-T110		1.53	244±5	1.12±0.01	273±5	96±4	4±2
JC-205	Suc-T110, RBSL3-*dcuB*	1.82	231±5	1.25±0.03	295±5	64±5	3±1
JC-206	Suc-T110, RBSL4-*dcuB*	1.94	243±5	1.15±0.01	278±5	82±5	3±2
JC-207	Suc-T110, RBSL2-dcuC	1.51	245±5	1.32±0.04	324±4	84±5	3±1
JC-208	Suc-T110, RBSL3-dcuC	1.36	245±3	1.25±0.02	311±4	94±5	2±1
JC-209	Suc-T110, RBSL3-*dcuB*, RBSL2-*dcuC*	1.65	261±1	1.22±0.01	321±4	80±6	1±1
JC-210	Suc-T110, RBSL3-*dcuB*, RBSL3-*dcuC*	1.50	250±1	1.21±0.01	305±1	75±5	2±1
JC-211	Suc-T110, RBSL4-*dcuB*, RBSL2-*dcuC*	1.69	257±4	1.41±0.02	366±4	90±7	1±0
JC-212	Suc-T110, RBSL4-*dcuB*, RBSL3-*dcuC*	1.68	253±1	1.24±0.01	315±1	65±1	1±1

[a] Fermentations were carried out in NBS mineral salts medium containing 5 % glucose and 100 mM potassium bicarbonate for 72 h. The pH was maintained at 7.0 by automatic addition of a base containing 2.4 M potassium carbonate and 1.2 M potassium hydroxide

[b] The 5′ RBS sequences of *dcuB* gene of strains JC-205 and JC-206 were CAGGAGAGATAGA and CAGGAGGAACTAG. The 5′ RBS sequences of *dcuC* gene of strains JC-207 and JC-208 were CAGGAGATTGCGA and CAGGAGGACTAAG
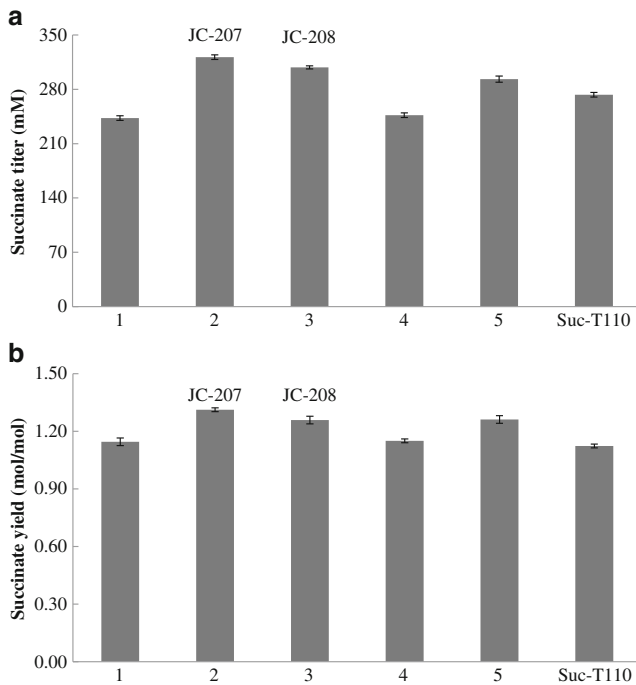
Fig. 4 Production of succinate by five colonies from the RBS library of *dcuC* gene. **a** Succinate titer; **b** succinate yield

yield were 10 and 40 % of the parent strain Suc-T110, respectively (Table 3). It was thus suggested that DcuB and DcuC were the main two transporters for succinate efflux during glucose fermentation.

Strength variations of RBS library

RBS library, which had a sequence of CAGGAGRNNN NNN in front of the ATG translation start site, was selected in this work for modulating *dcuB* and *dcuC* genes to improve succinate efflux capability. In order to test the strength variations of the RBS library, it was firstly constructed in front of *lacZ* gene in the chromosome of *E. coli* ATCC 8739. After recombination, β-galactosidase activities of 29 colonies verified by PCR amplification and wild-type stain ATCC 8739 were measured. Strengths of regulatory parts in the RBS library distributed evenly (Fig. 2), which varied from 0.17 to 8.6 times of induced *lacZ* promoter. It was suggested that RBS library used in this work could lead to a wide range of strength variation.

Modulating *dcuB* and *dcuC* genes with RBS library

A regulatory part library, which included a promoter, a mRNA stabilizing region and a RBS library (Fig. 1a) was inserted in the ATG translation start site of *dcuB* and *dcuC* genes. The native promoters of *dcuB* and *dcuC* genes were kept. After

modulating *dcuB* gene with RBS library, five strains verified by PCR were randomly picked for glucose fermentation (Fig. 3). Succinate titers varied from 235 to 295 mM and succinate yields varied from 1.12 to 1.25 mol/mol in these five strains. Strain Suc-T110-dcuB3, which had the highest succinate titer and yield, was renamed JC-205 (Table 4). Succinate titer increased 8 % compared to parent strain Suc-T110. Another strain Suc-T110-dcuB4, which produced 278 mM succinate with a yield of 1.15 mol/mol, was renamed JC-206 (Table 4).

Five strains verified by PCR in the *dcuC* library were also picked for glucose fermentation (Fig. 4). Succinate titers varied from 243 to 324 mM and succinate yields varied from 1.15 to 1.32 mol/mol in these five strains. Strain Suc-T110-dcuC2, which had the highest succinate titer and yield, was renamed JC-207 (Table 4). Succinate titer increased 19 % compared to parent strain Suc-T110. Another strain Suc-T110-dcuC3, which produced 311 mM succinate with a yield of 1.25 mol/mol, was renamed JC-208 (Table 4).

Modulating *dcuB* and *dcuC* genes in combination to improve succinate production

The *dcuB* and *dcuC* genes were further modulated in combination to improve succinate production. The *dcuC* gene of strains JC-205 and JC-206 were modulated with RBSL2 and RBSL3, resulting in strains JC-209, JC-210, JC-211, and JC-212 (Table 1). Succinate titer and yield increased in all four modulated strains compared to parent strains JC-205 or JC-206 (Table 4). The best strain JC-211, which had *dcuB* gene modulated with RBSL4 and *dcuC* gene modulated with RBSL2, produced 366 mM succinate with a yield of 1.41 mol/mol. Succinate titer of strain JC-211 was 32 % higher than the parent strain JC-206 and 34 % higher than unmodulated strain Suc-T110 (Table 4). On the other hand, succinate yield of strain JC-211 was 23 % higher than the parent strain JC-206 and 26 % higher than unmodulated strain Suc-T110 (Table 4).

Discussions

Modulating gene expression directly in chromosome by multiple regulatory parts with varied strengths had been demonstrated to be better than plasmid-based gene overexpression using inducible strong promoters (Shi et al. 2013; Tan et al. 2013; Zhao et al. 2013). However, it has been suggested that expression level of a specific gene depended on both the sequence of regulatory part and sequence of gene itself (Shi et al. 2013) since different gene sequences would have an influence on the secondary structures between the transcription and translation start sites (Carrier and Keasling 1999). Thus, regulatory parts having high strengths for one gene

(such as *lacZ* gene) might have low strengths for other genes. Modulating gene expression with a regulatory part library would solve this problem since a wide range of strengths would be obtained. In this work, RBS library constructed in front of *lacZ* gene exhibited a strength variation of 50 times. The highest strength was 8.6 times of induced *E. coli lacZ* promoter, which was 72 % higher than commonly used T7 promoter (five times of induced *E. coli lacZ* promoter).

Product efflux system is very important for efficient production of target compounds. Although *E. coli* contains multiple Dcu carries for C_4-dicarboxylates transportation, only DcuC had been reported previously to be involved in succinate efflux during glucose fermentation (Zientz et al. 1999). The main function of DcuB had been reported to catalyze C_4-dicarboxylate exchange, for instance, fumarate uptake in exchange for succinate efflux (Six et al. 1994). In addition, DcuB had been implied in succinate efflux as well (Six et al. 1994). In this study, through deletion of four *dcu* genes either individually or in combination, it was found that both DcuB and DcuC were important for succinate efflux during glucose fermentation. Although deleting *dcuB* or *dcuC* gene individually led to only a small decrease of succinate production, deleting these two genes in combination had a 90 % decrease of succinate titer. It was suggested that DcuB and DcuC function as independent and mutually redundant succinate efflux transporters during glucose fermentation.

In a previous report, it was found that succinate efflux during glucose fermentation was not affected in an *E. coli* mutant deficient in DctA, DcuA, DcuB, DcuC, and DcuD (Janausch et al. 2001), suggesting that a carrier different from, or in addition to, the known Dcu and CitT carriers was used for succinate efflux. This was different with the finding in this work. It should be noted that only 1 mM succinate was produced in the previous *E. coli* mutant (Janausch et al. 2001). In contrast, the starting *E. coli* strain we used in this work produced 273 mM succinate in 72 h. The adverse results were suggested to be caused by the different succinate-producing level in these two strains. The unknown carrier can replace the succinate efflux function of Dcu carriers in a low intracellular succinate concentration, while it cannot replace the function of DcuB and DcuC when large amounts of succinate need to be excreted.

Furthermore, through modulating *dcuB* and *dcuC* genes in combination, succinate titer increased from 273 to 366 mM and succinate yield increased from 1.12 to 1.41 mol/mol. To the best of knowledge, this was the first report about improving succinate production through engineering succinate efflux system.

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