

Metabolic Engineering of *Escherichia coli* for Enhanced Production of Succinic Acid, Based on Genome Comparison and In Silico Gene Knockout Simulation

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Received 19 May 2005/Accepted 15 August 2005

Comparative analysis of the genomes of mixed-acid-fermenting *Escherichia coli* and succinic acid-overproducing *Mannheimia succiniciproducens* was carried out to identify candidate genes to be manipulated for overproducing succinic acid in *E. coli*. This resulted in the identification of five genes or operons, including *ptsG*, *pykF*, *sdhA*, *mgo*, and *aceBA*, which may drive metabolic fluxes away from succinic acid formation in the central metabolic pathway of *E. coli*. However, combinatorial disruption of these rationally selected genes did not allow enhanced succinic acid production in *E. coli*. Therefore, in silico metabolic analysis based on linear programming was carried out to evaluate the correlation between the maximum biomass and succinic acid production for various combinatorial knockout strains. This in silico analysis predicted that disrupting the genes for three pyruvate forming enzymes, *ptsG*, *pykF*, and *pykA*, allows enhanced succinic acid production. Indeed, this triple mutation increased the succinic acid production by more than sevenfold and the ratio of succinic acid to fermentation products by ninefold. It could be concluded that reducing the metabolic flux to pyruvate is crucial to achieve efficient succinic acid production in *E. coli*. These results suggest that the comparative genome analysis combined with in silico metabolic analysis can be an efficient way of developing strategies for strain improvement.

Succinic acid is one of the fermentation products of anaerobic metabolism as well as an intermediate of the tricarboxylic acid cycle. It has been used as a precursor for various chemicals, a food additive, an ion chelator, and a supplement to pharmaceuticals (34). Succinic acid has mostly been produced chemically from maleic anhydride. Recently, fermentative production of succinic acid has been receiving much research attention, as several bacteria can produce succinic acid as a major fermentation product. The naturally isolated obligate anaerobe *Anaerobiospirillum succiniciproducens* (6) and facultative anaerobes belonging to the family *Pasteurellaceae*, such as *Actinobacillus succinogenes* (10) and *Mannheimia succiniciproducens* (16), have been shown to be able to produce succinic acid efficiently.

Escherichia coli also produces succinic acid but as a minor fermentation product. *E. coli* prefers to produce much more acetic acid, formic acid, lactic acid, and ethanol rather than succinic acid during anaerobic fermentation. Thus, it is necessary to redirect metabolic fluxes for increasing succinic acid production as well as reducing formation of other metabolites. Toward this goal, an *ldhA* and *pfl* double mutant *E. coli* strain

NZN111 was developed to block the formation of lactic, acetic, and formic acids (2). However, cell growth was relatively slow, possibly due to the inactivation of enzymes involved in pyruvate dissimilation (3). Chatterjee and coworkers (4) reported that an additional *ptsG* gene mutation recovered the growth of NZN111 to some extent. However, acetic acid, formic acid, and ethanol were still formed. Amplification of CO₂-fixing anaerobic pathways catalyzed by phosphoenolpyruvate (PEP) carboxylase (18), malic enzyme (12, 27, 28), and pyruvate carboxylase (9, 31) have resulted in the enhancement of succinic acid production in *E. coli*. Recently, Kim et al. (14) reported that amplification of the *Actinobacillus succinogenes* PEP carboxylase in a PEP carboxylase-negative *E. coli* strain increased succinic acid production. Lin et al. (17) showed that succinic acid could be aerobically produced by utilizing the glyoxylate cycle in *E. coli*. Also, Sanchez et al. (23) reported impressive results for anaerobic production of succinic acid with a cumulative yield of 160 mM of succinic acid from 100 mM glucose in 24 h by repeated feeding fermentation of recombinant *E. coli*.

We recently reported the complete genome sequence of *M. succiniciproducens* MBEL55E, a gram-negative capnophilic bacterium, which was isolated from bovine rumen (16), and explored the genome-scale metabolic characteristics leading to high-level succinic acid production (11). It was reasoned that efficient production of succinic acid by *E. coli* would be possible by engineering its metabolic pathways to mimic those of *M. succiniciproducens*.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference
<i>E. coli</i> strains		
W3110	Coli Genetic Stock Center strain (CGSC) no. 4474	CGSC
W3110G	<i>ptsG::Sp^r</i>	This study
W3110GF	<i>ptsG::Sp^r pykF::Tc^r</i>	This study
W3110GFO	<i>ptsG::Sp^r pykF::Tc^r mqo::Cm^r</i>	This study
W3110GFH	<i>ptsG::Sp^r pykF::Tc^r sdh::Km^r</i>	This study
W3110GFHO	<i>ptsG::Sp^r pykF::Tc^r sdh::Km^r mqo::Cm^r</i>	This study
W3110GFHOE	<i>ptsG::Sp^r pykF::Tc^r sdh::Km^r mqo::Cm^r aceBA::Pm^r</i>	This study
W3110GFA	<i>ptsG::Sp^r pykF::Tc^r pykA::Km^r</i>	This study
W3110GFAP	<i>ptsG::Sp^r pykF::Tc^r pykA::Km^r pflB::Cm^r</i>	This study
W3110GFAPL	<i>ptsG::Sp^r pykF::Tc^r pykA::Km^r pflB::Cm^r ldhA::Pm^r</i>	This study
Plasmids		
pKD46	Red recombinase expression plasmid, temperature-sensitive <i>ori</i> ; Ap ^r	5
pACYC184	Cm ^r Tc ^r	New England Biolabs ^b
pIC156	Sp ^r	26
pUC19-phleo	Ap ^r Pm ^r	7
pUC4K	Ap ^r Km ^r	Pharmacia ^c

^a Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Pm, phleomycin; Sp, spectinomycin; Tc, tetracycline.

^b New England Biolabs, Inc., Beverly, Mass.

^c Pharmacia Biotech, Uppsala, Sweden.

In this paper, we report comparative analysis of the central metabolic pathways of *M. succiniciproducens* and *E. coli* to select important metabolic pathways with respect to the formation of fermentation products. Subsequent in silico analysis was performed to predict the target genes among the candi-

dates to be removed from *E. coli* for the efficient production of succinic acid. Finally, these predictions were validated by actual gene knockout experiments. In addition, the reasons for different fermentation characteristics between the rumen bacterium and *E. coli* are discussed.

TABLE 2. Oligonucleotides used for gene replacement experiments

Template	PCR no.	Primer	Sequence (5' → 3')
SpR	1	PTSG1	TGC CCG CCG TTG TAT CGC ATG TTA TGG CAG GGG GAT CGA TCC TCT AGA
		PTSG2	TGC AGC AAC CAG AGC CGG TGC CAT TTC GCT GGG CCG ACA GGC TTT
	2	PTSG3	TGG GCG TCG GTT CCG CGA ATT TCA GCT GGC TGC CCG CCG TTG TAT CGC
		PTSG4	GAG GTT AGT AAT GTT TTC TTT ACC ACC AAA TGC AGC AAC CAG AGC CGG
TcR	1	PYKF1	TGG ACG CTG GCA TGA ACG TTA TGC GTC TGA GGG TAG ATT TCA GTG CAA
		PYKF2	CGC CTT TGC TCA GTA CCA ACT GAT GAG CCG GGG TTC CAT TCA GGT CGA
	2	PYKF3	CCG AAT CTG AAG AGA TGT TAG CTA AAA TGC TGG ACG CTG GCA TGA ACG
		PYKF4	AAG TGA TCT CTT TAA CAA GCT GCG GCA CAA CGC CTT TGC TCA GTA CCA
CmR	1	MQO1	GGC ATA CCA TGC CGG ATG TGG CGT ATC ATT GGG GTT TAA GGG CAC CAA
		MQO2	GAA CTA CGG CGA GAT CAC CCG CCA GTT AAT GCC CCG GGC TTT GCG CCG
	2	MQO3	TGG CGC GTC TTA TCA GCA TAC GCC ACA TCC GGC ATA CCA TGC CGG ATG
		MQO4	AGC CAC GCG TAC GGA AAT TGG TAC CGA TGT GAA CTA CGG CGA GAT CAC
KmR	1	SDH1	CAG TCA GAG AAT TTG ATG CAG TTG TGA TTG ATC GGG GGG GGG GGA AAG
		SDH2	ATC GGC TCT TTC ACC GGA TCG ACG TGA GCG ATC CCA ATT CTG ATT AGA
	2	SDH3	GTT GTG GTG TGG GGT GTG TGA TGA AAT TGC CAG TCA GAG AAT TTG ATG
		SDH4	ATC ATG TAG TGA CAG GTT GGG ATA ACC GGA ATC GGC TCT TTC ACC GGA
PmR	1	ACEBA1	GCA CCT TGT GAT GGT GAA CGC ACC GAA GAA CGA GCT CGG TAC CCG GGC
		ACEBA2	CTT TCG CCT GTT GCA GCG CCT GAC CGC CAG CAA TAG ACC AGT TGC AAT
	2	ACEBA3	GAC GCG CCG ATT ACT GCC GAT CAG CTG CTG GCA CCT TGT GAT GGT GAA
		ACEBA4	ATC CCG ACA GAT AGA CTG CTT CAA TAC CCG CTT TCG CCT GTT GCA GCG
KmR	1	PYKA1	CAC CTG GTT GTT TCA GTC AAC GGA GTA TTA CAT CGG GGG GGG GGG AAA G
		PYKA2	GTG GCG TTT TCG CCG CAT CCG GCA ACG TAC ATC CCA ATT CTG ATT AG
	2	PYKA3	TTA TTT CAT TCG GAT TTC ATG TTC AAG CAA CAC CTG GTT GTT TCA GTC
		PYKA4	GTT GAA CTA TCA TTG AAC TGT AGG CCG GAT GTG GCG TTT TCG CCG CAT C
PmR	1	LDHA1	CGG TGT GGC GAT GCT GCG CAT TCT GAA AGG CAA TAG ACC AGT TGC AAT
		LDHA2	GTC AGA GCT TCT GCT GTC AGG AAT GCC TGG CGA GCT CGG TAC CCG GGC
	2	LDHA3	AAC GGC AGG CGT TAT CGG TAC CGG TAA AAT CGG TGT GGC GAT GCT GCG
		LDHA4	AAG TTT TGC AGC GTA GTC TGA GAA ATA CTG GTC AGA GCT TCT GCT GTC
CmR	1	PFLB1	GGG GTG TTA CCC GTT TTC TTA CCA TAC ACA GGG GTT TAA GGG CAC CAA
		PFLB2	AGT GGA CTT ACT TCG GCT ACC TGG CTG CTG GGG CTT TGC GCC GAA TAA
	2	PFLB3	CCG AAC GGC GCG CCA GCA CGA CGA CCG TCT GGG GTG TTA CCC GTT TTC
		PFLB4	GTC CGG CTA CCA ACG CTC AGG AAG CTA TCC AGT GGA CTT ACT TCG GCT

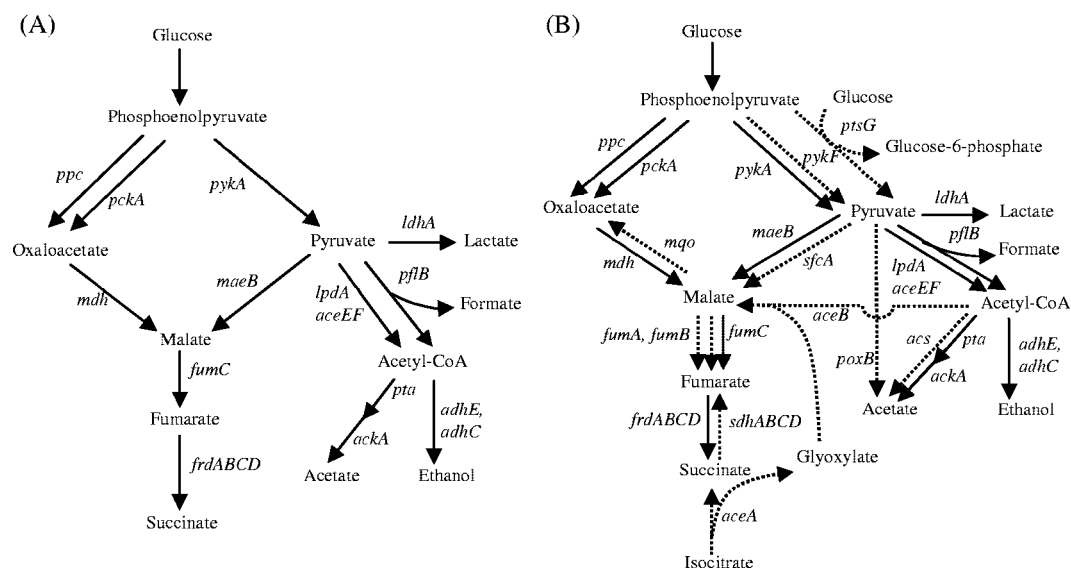


FIG. 1. Comparison of the central metabolic pathways related to succinic acid formation in *M. succiniciproducens* (A) and *E. coli* (B). Gene names are shown in italics. Dashed arrows in panel B represent those metabolic pathways only existing in *E. coli*. Acetyl-coenzyme A (Acetyl-CoA) synthetase (*acs*), fumarase AB (*fumAB*), glyoxylate shunt (*aceBA*), malate dehydrogenase (*mqo*), NAD-dependent malic enzyme (*sfcA*), phosphotransferase system (*ptsG*), pyruvate kinase F (*pykF*), pyruvate oxidase (*poxB*), and succinate dehydrogenase (*sdhABCD*) genes exist in *E. coli* but not in *M. succiniciproducens*.

MATERIALS AND METHODS

Strains and plasmids. All strains and plasmids used in this study are listed in Table 1. All DNA manipulations were carried out by following standard protocols (22). Plasmid pKD46 (5), harboring the bacteriophage λ *red* operon, which contains the *exo*, *beta*, and *gam* genes, was used for disrupting the genes of interest in the chromosome of *E. coli* W3110 with appropriate antibiotic markers (Table 2). Recombinant *E. coli* W3110 harboring pKD46 was cultivated at 30°C, and λ recombinases were induced by adding L-arabinose (1 mM) at an optical density at 600 nm (OD_{600}) of 0.4. Then electrocompetent cells were prepared by standard protocol (22). Two-step PCRs were performed using the antibiotic resistance genes as templates and primers listed in Table 2. The PCR products were transformed into the electrocompetent *E. coli* W3110 harboring pKD46. Colonies were selected on Luria-Bertani (LB; 10 g tryptone liter⁻¹, 5 g yeast extract liter⁻¹, 10 g NaCl liter⁻¹) agar plates containing the appropriate antibiotics at the following concentrations (μ g ml⁻¹): chloramphenicol, 34; kanamycin, 25; phleomycin, 5; tetracycline, 15; spectinomycin, 50. Successful gene replacement with the antibiotic marker was confirmed by PCR.

Comparative genome analysis. The central metabolic pathways of *E. coli* K-12 were reconstructed using the information extracted from the metabolic database BioSilico (13). The fermentation pathways of *M. succiniciproducens* MBEL55E were reconstructed based on the recent genome annotation (<http://www.ncbi.nlm.nih.gov>). Two reconstructed metabolic pathways were compared to select the target genes in *E. coli* to be manipulated for mimicking efficient succinic acid production in *M. succiniciproducens*.

Glucose phosphorylation assay. To elucidate whether *M. succiniciproducens* uses a PEP-sugar phosphotransferase system (PTS) to actively uptake glucose, ¹⁴C-labeled glucose phosphorylation assay was carried out as described previously (25). Cell extracts of *E. coli* W3110 and *M. succiniciproducens* MBEL55E were employed for the assays using ¹⁴C-labeled glucose as a substrate and PEP or ATP as a phosphate donor. The amounts of phosphorylated glucose passed through a polystyrene column were analyzed by a scintillation counter.

In silico analysis. A genome-scale in silico *E. coli* model (8, 21) was used with slight modifications. The specific growth rate can be calculated by a biomass equation derived from the drain of biosynthetic precursors into *E. coli* biomass with their appropriate ratios (19). Under the pseudo-steady-state assumption, the unknown internal fluxes within the underdetermined metabolic reaction network can be evaluated by means of linear programming subject to the constraints provided by mass conservation, thermodynamics, and reaction stoichiometry (8, 24, 30). The in silico analysis was carried out using the MetaFluxNet program package, version 1.69 (15).

Culture conditions. Cells were routinely cultured in LB medium at 37°C. Antibiotics were added at the concentrations shown above depending on the knockout markers used. For the fermentation experiments, cells were first grown in 10 ml LB medium at 37°C with shaking. One milliliter of seed culture was used to inoculate a 125-ml butyl rubber-stoppered serum vial containing 100 ml of fermentation medium, which contains (per liter): glucose, 9 g (50 mM); yeast extract, 5 g; NaHCO₃, 10 g; NaH₂PO₄ · H₂O, 8.5 g; K₂HPO₄, 15.5 g (pH 7.0). The vial headspace was filled with CO₂, and Na₂S · 9H₂O was added to a final concentration of 1 mg liter⁻¹ to ensure anaerobic conditions.

Analytical procedure. For the measurement of the concentrations of glucose and organic acids, culture supernatant was passed through a syringe filter (pore size of 0.2 μ m) after centrifugation at 10,000 \times g for 10 min. The glucose concentration was determined by using a glucose analyzer (model 2300 STAT; Yellow Springs Instrument Co., Yellow Springs, Ohio). The concentrations of organic acids were determined by high-performance liquid chromatography (L-3300 RI monitor, L-4200 UV-VIS detector, D2500 chromato-integrator; Hitachi, Tokyo, Japan) equipped with an Aminex HPLC-87H column (300 by 7.8 mm, Hercules, Calif.). The column was eluted isocratically at 50°C at a flow rate of 0.6 ml/min with 0.01 N H₂SO₄. Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}) using an Ultrospec3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

RESULTS

Comparison of central metabolic pathways of *E. coli* and *M. succiniciproducens*. *M. succiniciproducens* has been reported to produce succinic acid very efficiently (11). On the other hand, succinic acid is a minor product of mixed-acid fermentation in *E. coli*. It was reasoned that production of succinic acid in *E. coli* would be possibly enhanced by mimicking the metabolism of *M. succiniciproducens*. Toward this goal, we compared the central metabolic pathways of *E. coli* and *M. succiniciproducens*, aiming to identify candidate metabolic genes that possibly determine the different fermentation patterns in the two bacteria. Considering that the genome size of *E. coli* is about twice as large as that of *M. succiniciproducens*, we focused on metabolic pathways that are found in *E. coli* but not in *M. succi-*

niciproducens, since they were thought to drive metabolic flux away from succinic acid formation in *E. coli*. Compared in Fig. 1, succinate dehydrogenase (*sdhABCD*), malate dehydrogenase (*mgo*), glyoxylate shunt (*aceBA*), and *ptsG* homologue genes were found in *E. coli* but not in *M. succiniciproducens*. While *E. coli* has two pyruvate kinases, A and F, encoded by the *pykA* and *pykF* genes, *M. succiniciproducens* has pyruvate kinase A only.

Even though the *sfcA*, *fumAB*, *poxB*, and *acs* genes were only present in *E. coli*, they were not considered as knockout candidates for the following reasons. The malic enzyme encoded by the *sfcA* gene has been reported to be beneficial for succinic acid production (12, 27). The fumarases encoded by the *fumAB* genes do not seem to negatively affect succinic acid production because *fumA* expression is strongly repressed under anaerobic conditions (32), while FumB generates fumarate for use as an anaerobic electron acceptor (33). The products of the *poxB* and *acs* genes play important roles in efficient aerobic growth (1) and growth on acetic acid (20), respectively, and thus do not seem to affect succinic acid production under anaerobic conditions. Therefore, we selected five genes or operons, *ptsG*, *pykF*, *mgo*, *sdhABCD*, and *aceBA*, as the initial targets to be inactivated to make *E. coli* mimic *M. succiniciproducens*.

Comparative metabolic engineering. Comparative engineering of *E. coli* metabolic pathways was performed based on the above findings. The *ptsG*, *pykF*, *mgo*, *sdhABCD*, and *aceBA* genes in *E. coli*, which were not found in the genome of *M. succiniciproducens*, were sequentially disrupted to examine their effects on fermentation profiles. The mutant strains constructed are listed in Table 1. First, the *ptsG* and *pykF* genes involved in pyruvate formation were inactivated, hoping that more PEP will be available for the carboxylation reaction. Then, the *mgo* and *sdhA* genes were sequentially deleted to prevent the reverse flux from succinic acid. Finally, the *aceBA* genes responsible for the glyoxylate shunt were inactivated.

Anaerobic fermentation profiles of these mutant strains are summarized in Table 3. Interestingly, mutants rationally constructed by comparative metabolic engineering did not change the fermentation profiles much compared with the wild-type strain. Even the most heavily engineered W3110GFOHE strain, which was designed to be the strain most similar to *M. succiniciproducens*, did not show a significant difference in the growth rate and fermentation patterns compared with the wild-type strain (Table 3). Metabolic engineering based only on genome and pathway comparison was not successful, probably because we did not examine all possible combinational mutations of the candidate reactions. Since it is practically impossible to construct all these possible combinatorial mutant strains, in silico knockout experiments were carried out.

In silico prediction of gene candidates for enhanced succinic acid production. The effects of combinatorial gene knockouts on succinic acid formation were examined in silico using the genome-scale *E. coli* metabolic model (21). The maximum biomass formation rate was used as an objective function during the optimization. When the enzymes (genes) were knocked out, the fluxes of the corresponding metabolic reactions were set to zero. It should also be noted that the metabolic reactions catalyzed by the pyruvate kinases A and F cannot be distin-

TABLE 3. Results of anaerobic fermentation of *E. coli* W3110 and its various mutant strains

Strain	Fermentation time (h)	OD ₆₀₀	Concn of substrate or product (mM) ^a							Succinic acid ratio ^c	Fold change ^d
			Glucose ^b	Succinic acid	Lactic acid	Formic acid	Acetic acid	Ethanol			
W3110	24	1.79 ± 0.11	5.07 ± 0.45	2.43 ± 0.03	10.62 ± 2.42	88.03 ± 0.42	40.10 ± 0.20	5.77 ± 0.06	0.017	1.00	
Strains constructed based on metabolic pathway comparison											
W3110G	24	1.47 ± 0.01	5.66 ± 1.77	2.16 ± 0.08	13.71 ± 3.46	88.57 ± 2.97	40.69 ± 0.97	5.98 ± 0.09	0.014	0.86	
W3110GF	24	1.46 ± 0.04	4.28 ± 1.42	2.83 ± 0.07	14.13 ± 1.32	86.25 ± 2.02	40.10 ± 0.58	5.92 ± 0.24	0.019	1.15	
W3110GFO	24	1.49 ± 0.07	4.68 ± 0.35	2.67 ± 0.33	12.97 ± 0.06	88.39 ± 0.81	40.89 ± 0.18	6.00 ± 0.08	0.018	1.07	
W3110GFH	24	1.35 ± 0.01	4.70 ± 0.39	2.51 ± 0.02	15.18 ± 1.49	85.86 ± 0.10	38.88 ± 0.16	5.84 ± 0.06	0.017	1.02	
W3110GFOH	24	1.28 ± 0.11	4.82 ± 0.48	2.58 ± 0.03	17.06 ± 0.45	90.40 ± 2.55	40.40 ± 0.49	6.20 ± 0.06	0.016	1.00	
W3110GFOHE	24	1.27 ± 0.05	4.25 ± 0.27	2.49 ± 0.18	13.31 ± 0.78	85.98 ± 0.38	38.66 ± 0.02	5.84 ± 0.01	0.017	1.03	
Strains constructed based on in silico simulation results											
W3110GFFA	24	0.73 ± 0.06	20.57 ± 3.02	8.16 ± 0.01	5.47 ± 0.49	27.47 ± 2.94	16.48 ± 1.55	1.88 ± 0.24	0.137	8.29	
W3110GFBA	80	0.99 ± 0.04	0.41 ± 0.05	17.35 ± 0.03	10.67 ± 0.53	50.36 ± 3.00	30.25 ± 1.47	4.14 ± 0.42	0.150	9.23	
W3110GFAP	80	0.682 ± 0.16	12.2 ± 0.04	8.45 ± 0.05	21.4 ± 0.88	ND ^e	4.1 ± 0.04	5.5 ± 0.33	0.214	12.60	
W3110GFAPL	80	0.05 ± 0.02	41.6 ± 0.8	0.39 ± 0.01	ND	ND	ND	1.6 ± 0.07	0.19	11.53	

^a Anaerobic vial fermentation on 50 mM initial glucose for 24 or 80 h.

^b Residual glucose concentration.

^c Calculated as succinic acid/(succinic acid + lactic acid + formic acid + acetic acid + ethanol).

^d Calculated as succinic acid ratio/succinic acid ratio in the wild-type strain (0.017).

^e Not detected.

TABLE 4. Prediction of maximum biomass formation rate, succinic acid production rate, and succinic acid flux ratio in various in silico *E. coli* mutant strains

In silico strain	Disrupted gene(s)	Maximum biomass formation rate (h ⁻¹)	Succinic acid production rate (mmole g dry cell weight ⁻¹ h ⁻¹)	Succinic acid flux ratio ^a
Wild type		0.2163	0.0687	0.0020
H	<i>sdhA</i>	0.2163	0.0687	0.0020
O	<i>mgo</i>	0.2163	0.0687	0.0020
E	<i>aceBA</i>	0.2163	0.0687	0.0020
G	<i>ptsG</i>	0.1891	0.0601	0.0017
FA	<i>pykFA</i>	0.2163	0.0687	0.0020
HO	<i>sdhA mgo</i>	0.2163	0.0687	0.0020
HE	<i>sdhA aceBA</i>	0.2163	0.0687	0.0020
HG	<i>sdhA ptsG</i>	0.1891	0.0601	0.0017
HFA	<i>sdhA pykFA</i>	0.2163	0.0687	0.0020
OE	<i>mgo aceBA</i>	0.2163	0.0687	0.0020
OG	<i>mgo ptsG</i>	0.1891	0.0601	0.0017
OFA	<i>mgo pykFA</i>	0.2163	0.0687	0.0020
EG	<i>aceBA ptsG</i>	0.1891	0.0601	0.0017
EFA	<i>aceBA pykFA</i>	0.2163	0.0687	0.0020
GFA	<i>ptsG pykFA</i>	0.1366	6.8339	0.2311
HOE	<i>sdhA mgo aceBA</i>	0.2163	0.0687	0.0020
HOG	<i>sdhA mgo ptsG</i>	0.1891	0.0601	0.0017
HOFA	<i>sdhA mgo pykFA</i>	0.2163	0.0687	0.0020
HEG	<i>sdhA aceBA ptsG</i>	0.1891	0.0601	0.0017
HEFA	<i>sdhA aceBA pykFA</i>	0.2163	0.0687	0.0020
HGFA	<i>sdhA ptsG pykFA</i>	0.1366	6.8339	0.2311
OEG	<i>mgo aceBA ptsG</i>	0.1891	0.0601	0.0017
OGFA	<i>mgo ptsG pykFA</i>	0.1366	6.8339	0.2311
OEFA	<i>mgo aceBA pykFA</i>	0.2163	0.0687	0.0020
EGFA	<i>aceBA ptsG pykFA</i>	0.1366	6.8339	0.2311
HOEG	<i>sdhA mgo aceBA ptsG</i>	0.1891	0.0601	0.0017
HOEFA	<i>sdhA mgo aceBA pykFA</i>	0.2163	0.0687	0.0020
HOGFA	<i>sdhA mgo ptsG pykFA</i>	0.1366	6.8339	0.2311
HEGFA	<i>sdhA aceBA ptsG pykFA</i>	0.1366	6.8339	0.2311
OEGFA	<i>mgo aceBA ptsG pykFA</i>	0.1366	6.8339	0.2311
HOEGFA	<i>sdhA mgo aceBA ptsG pykFA</i>	0.1366	6.8339	0.2311
GFAP	<i>ptsG pykFA pfl</i>	0.1219	9.1236	0.5034
GFAPL	<i>ptsG pykFA pfl ldhA</i>	0.1219	9.1236	0.5034

^a Calculated as (succinic acid formation flux)/(summed formation fluxes of succinic acid, lactic acid, formic acid, acetic acid, and ethanol).

guished in the in silico *E. coli* metabolic model because they catalyze the same reaction. The results of in silico experiments for the representative mutants are shown in Table 4.

It was found that single-gene knockouts and many multiple-gene knockouts did not significantly change the succinic acid production. The succinic acid production rate was increased by 100-fold when the *ptsG* and *pykFA* genes were knocked out in silico. Further knockout of the *sdhA*, *mgo*, and *aceBA* genes or their combinations did not increase the succinic acid production rate. However, inactivation of the *pfl* gene further increased the succinic acid production rate (Table 4). In conclusion, the in silico simulation results suggested that three pyruvate-forming enzymes encoded by the *ptsG* and *pykFA* genes need to be inactivated for the enhanced production of succinic acid in *E. coli*, which can be further increased by inactivating the *pfl* gene.

Validation of in silico prediction. According to the in silico prediction, *E. coli* W3110GFA was constructed by knocking out the *ptsG* and *pykFA* genes. Anaerobic fermentation of

W3110GFA for 24 h resulted in an about 3.4-fold increase in succinic acid formation (from less glucose consumed) and a significant reduction of other fermentation products compared with the wild-type strain (Table 3). The succinic acid ratio increased by more than eightfold using the mutant strain. In 80 h, *E. coli* W3110GFA was able to convert 50 mmol of glucose to 17.4 mmol of succinic acid, which is more than 7 times higher than that produced by wild-type and other mutant strains constructed based on pathway comparison. It can also be seen that the formation of other fermentation products was considerably reduced in W3110GFA, resulting in the 9.23-fold increase in succinic acid ratio compared with the wild-type strain (Table 3). These results suggest that the anaerobic fermentation pattern of *E. coli* could be dramatically affected by simultaneous disruption of strong pyruvate-forming enzymes present in *E. coli* as predicted by in silico analysis. It also indicates that blocking the major pathways converting PEP to pyruvate is beneficial in redirecting the metabolic flux to succinic acid in *E. coli*.

TABLE 5. Effects of external supply of pyruvate on the anaerobic fermentation profile of *E. coli* W3110GFA

Strain	Concn of initial carbon source (mM)		OD ₆₀₀	Concn of substrate or product (mM) ^a						Succinic acid ratio ^c	Fold ^d change
	Glucose	Pyruvate		Glucose ^b	Succinic acid	Lactic acid	Formic acid	Acetic acid	Ethanol		
W3110	50		1.79 ± 0.11	5.07 ± 0.45	2.43 ± 0.03	10.62 ± 2.42	88.03 ± 0.42	40.10 ± 0.20	5.77 ± 0.06	0.017	1.00
W3110GFA	50		0.73 ± 0.06	20.57 ± 3.02	8.16 ± 0.01	5.47 ± 0.49	27.47 ± 2.94	16.48 ± 1.55	1.88 ± 0.24	0.137	8.29
W3110GFA	25	50	1.40 ± 0.07	21.46 ± 2.07	3.28 ± 0.59	12.98 ± 0.51	41.02 ± 4.29	44.98 ± 1.31	5.50 ± 0.20	0.031	1.82
W3110GFA		100	1.62 ± 0.02	ND ^e	3.11 ± 0.05	14.66 ± 1.67	66.08 ± 3.51	80.43 ± 4.99	4.82 ± 1.24	0.018	1.06

^a Anaerobic vial fermentation for 24 h.

^b Residual glucose concentration.

^c Calculated as succinic acid/(succinic acid + lactic acid + formic acid + acetic acid + ethanol).

^d Calculated as succinic acid ratio/succinic acid ratio in the wild-type strain (0.017).

^e Not detected.

Further disruption of the *pfl* and *ldhA* genes in a W3110GFA strain. With an aim to further reduce other fermentation products, the *pfl* and *ldhA* genes were inactivated in a W3110GFA strain. An *E. coli* W3110GFAP strain lacking the *pfl* gene showed slower growth than its parental W3110GFA strain. Formic acid was not detected, and acetic acid formation was dramatically decreased. However, a twofold increase of lactic acid formation was observed. Even though the final succinic acid concentration obtained with W3110GFAP was less than half of that obtained with W3110GFA, the succinic acid molar ratio was increased by 12.6-fold (Table 3), which is consistent with the in silico prediction results (Table 4). The W3110GFAPL strain, additionally lacking the *ldh* gene, showed normal growth under aerobic conditions but only marginal growth under anaerobic conditions. It could utilize only a little glucose under anaerobic conditions because of the lack of major anaerobic respiratory pathways (Table 3). In the absence of Na₂S, slightly enhanced succinic acid formation (<2 mM) was observed in the anaerobic culture of W3110GFAPL. These results suggest that pyruvate-forming enzymes (encoded by the *ptsG*, *pykF*, and *pykA* genes) and pyruvate-dissimilating enzymes (encoded by the *ldhA* and *pfl* genes) play essential roles in succinic acid production by *E. coli*.

Pyruvate supply affects the fermentation profiles of a W3110GFA strain. To examine the effects of pyruvate on the anaerobic metabolism of *E. coli*, pyruvate was supplied externally during the fermentation of *E. coli* W3110GFA. It was found that the addition of glucose plus pyruvate in the medium changed the active succinic acid fermentation pattern of W3110GFA to the mixed-acid fermentation, as in the wild-type strain (Table 5). When pyruvate was added as a sole carbon source, significant amounts of formic and acetic acids were formed. Notably, the succinic acid ratio also returned to that of the wild-type strain by the addition of pyruvate. These results suggest that pyruvate is mainly converted to formic and acetic acids rather than succinic acid in the anaerobic fermentation of *E. coli*.

DISCUSSION

E. coli carries out mixed-acid fermentation under anaerobic conditions and produces much more acetic acid, formic acid, lactic acid, and ethanol than succinic acid. In contrast, a rumen bacterium, *M. succiniciproducens*, can produce succinic acid as a major fermentation product. It was therefore assumed that different fermentation patterns might be caused by different fermentation pathways in operation in two bacteria.

To make *E. coli* mimic the fermentation pattern of *M. succiniciproducens*, several *E. coli* mutants were constructed based on the results of pathway comparison (Table 1). The *ptsG*, *pykF*, *mgo*, *sdhABCD*, and *aceBA* genes were selected as the target genes to be disrupted. It should be mentioned that *M. succiniciproducens* does not operate the PTS. We have previously reported that *M. succiniciproducens* transports glucose by the PTS if we accept the annotation results obtained using the clusters of orthologous group database. On the other hand, the annotation results based on the nonredundant database suggest that *M. succiniciproducens* does not utilize the PTS for glucose uptake. Therefore, we carried out actual experiments and found that the *M. succiniciproducens* does not utilize the PTS. *E. coli* W3110 was able to efficiently phosphorylate glucose using PEP or ATP as a phosphate donor. On the other hand, *M. succiniciproducens* could efficiently transfer phosphate from ATP to glucose but rather poorly from PEP. This is the reason why the *E. coli ptsG* gene was chosen as one of the candidate genes to be knocked out. However, even the most heavily engineered W3110GFHOE strain did not show significant changes in the fermentation profiles, suggesting that the robust metabolism aided by many shunts and isoenzymes present in *E. coli* hinders the flux changes. Thus, metabolic engineering based on simple yet rational comparison of the two genome-scale metabolic pathways was not successful. This was thought to be because we could not examine all the possible combinatorial knockout mutant strains. Since it is practically impossible to generate all the possible combinatorial mutant strains, we decided to carry out in silico knockout experiments. However, it requires great computational power and a long time to perform in silico simulations for all possible combinations. It was thus invaluable to have the results of rational genome comparison, as we could focus the simulations on those genes that were found to be important for succinic acid production.

The results of in silico experiments suggested that knocking out the *ptsG*, *pykF*, and *pykA* genes is the most beneficial for the enhanced production of succinic acid. It is interesting to note that in silico *E. coli* cannot distinguish the products of the *pykF* and *pykA* genes, as they catalyze the same reaction. During the comparative metabolic engineering studies, the *pykA* gene was not considered a candidate gene to be knocked out because *M. succiniciproducens* also possessed this gene. It was, however, concluded from the in silico analysis that reducing

the pyruvate-forming flux is very important for enhanced succinic acid production. The actual experiments showed the predicted results. *E. coli* W3110GFA produced significantly more succinic acid and concomitantly less of other fermentation products, though it grew slightly slower than the wild-type strain under anaerobic conditions (Table 3). It is important to note that the metabolic fluxes could be altered to lead to enhanced succinic acid formation by manipulating the upstream pathways (e.g., the *ptsG*, *pykF*, and *pykA* genes) rather than the pathways directly involved in the end product formation (such as the *ldhA*, *pfl*, and *pta* genes). These findings are reasonable, as high-level succinic acid producers such as *M. succiniciproducens* and *A. succinogenes* efficiently convert PEP to succinic acid via oxaloacetate, malate, and fumarate (11, 29). Therefore, the metabolic flux to pyruvate is not beneficial for succinic acid production, since pyruvate is efficiently converted to other fermentation products in *E. coli*.

In a pyruvate complementation test, the formation of acetic and formic acids was significantly increased by the addition of pyruvate in the pyruvate-deficient W3110GFA strain (Table 5). Apparently, pyruvate is more readily converted to acetic and formic acids in *E. coli*. While *E. coli* has strong pathways for converting pyruvate ultimately to acetic acid (encoded by the *poxB*, *pta*, *ackA*, and *acs* genes) to generate ATP, *M. succiniciproducens* has relatively weak pyruvate-forming pathways, since it has no active PTS for glucose uptake and only one pyruvate kinase (compared with two in *E. coli*) as mentioned above. This is the reason why *M. succiniciproducens* can efficiently produce succinic acid via anaplerotic pathways while sustaining the low level of pyruvate. Therefore, pyruvate was found to be the most important central metabolite affecting, most significantly, the fermentation pattern of *E. coli*. This difference seems to determine the characteristic fermentation patterns of the two microorganisms.

In summary, successful metabolic engineering of *E. coli* for enhanced succinic acid production could be achieved by combining genome and pathway comparison, in silico metabolic characterization, and validation by knockout experiments. This strategy may be generally useful in improving microbial strains for the enhanced production of metabolites.

ACKNOWLEDGMENTS

We thank B. L. Wanner for providing plasmid pKD46 and E. Dervyn for plasmids pIC156 and pUC19-phleo.

This work was supported by the Genome-Based Integrated Bioprocess Project of the Ministry of Science and Technology and by the BK21 project. Further support by the LG Chem Chair Professorship, IBM-SUR program, Microsoft, and the Center for Ultramicrochemical Process Systems sponsored by KOSEF is appreciated.

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