ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Combining Metabolic Engineering and Metabolic Evolution to Develop Nonrecombinant Strains of *Escherichia coli* C That Produce Succinate and Malate

Kaemwich Jantama,[1] M.J. Haupt,[1] Spyros A. Svoronos,[1] Xueli Zhang,[2] J.C. Moore,[2] K.T. Shanmugam,[2] L.O. Ingram[2]

[1]Department of Chemical Engineering, University of Florida, Gainesville, Florida

[2]Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611; telephone: 352/392-8176; fax: 352/846-0969; e-mail: ingram@ufl.edu

Received 28 August 2007; revision received 9 October 2007; accepted 10 October 2007

Published online 30 October 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21694

ABSTRACT: Derivatives of *Escherichia coli* C were engineered to produce primarily succinate or malate in mineral salts media using simple fermentations (anaerobic stirred batch with pH control) without the addition of plasmids or foreign genes. This was done by a combination of gene deletions (genetic engineering) and metabolic evolution with over 2,000 generations of growth-based selection. After deletion of the central anaerobic fermentation genes (*ldhA*, *adhE*, *ackA*), the pathway for malate and succinate production remained as the primary route for the regeneration of NAD+. Under anaerobic conditions, ATP production for growth was obligately coupled to malate dehydrogenase and fumarate reductase by the requirement for NADH oxidation. Selecting strains for improved growth co-selected increased production of these dicarboxylic acids. Additional deletions were introduced as further improvements (*focA*, *pflB*, *poxB*, *mgsA*). The best succinate biocatalysts, strains KJ060(*ldhA*, *adhE*, *ackA*, *focA*, *pflB*) and KJ073(*ldhA*, *adhE*, *ackA*, *focA*, *pflB*, *mgsA*, *poxB*), produce 622–733 mM of succinate with molar yields of 1.2–1.6 per mole of metabolized glucose. The best malate biocatalyst, strain KJ071(*ldhA*, *adhE*, *ackA*, *focA*, *pflB*, *mgsA*), produced 516 mM malate with molar yields of 1.4 per mole of glucose metabolized.

Biotechnol. Bioeng. 2008;99: 1140–1153.

KEYWORDS: *Escherichia coli*; succinate; malate; metabolic evolution

Introduction

The fermentative production of succinate from renewable feedstocks will become increasingly competitive as petroleum prices increase. Succinate can serve as a substrate for transformation into plastics, solvents, and other chemicals currently made from petroleum (Lee et al., 2004; Lee et al., 2005; McKinlay et al., 2007; Wendisch et al., 2006; Zeikus et al., 1999). Many bacteria have been described with the natural ability to produce succinate as a major fermentation product (Guettler et al., 1998; Table I). Some of these such as *Actinobacillus succinogenes* (Guettler et al., 1996a,b; Meynial-Salles et al., 2007), *Anaerobiospirillum succiniciproducens* (Glassner and Datta, 1992), and *Mannheimia succiniciproducens* (Lee et al., 2006; Song et al., 2007) can produce at high rates (up to 4 g L^{-1} h^{-1}) with impressive titers of succinate (300–900 mM) and high yields (>1.1 mol succinate/mol glucose). However, these natural producers require complex media ingredients, which add cost associated with production, purification, and waste disposal.

A variety of genetic approaches have been used to engineer strains of *Escherichia coli* for succinate production with varying degrees of success (Table I). Again complex ingredients have been used in the media with these recombinants. Many succinate-producing strains have been developed by deleting competing pathways and over-expressing native genes using plasmids. Strain NZN111 was engineered by inactivating two genes (*pflB* encoding pyruvate-formate lyase and *ldhA* encoding lactate dehydrogenase), and over-expressing two *E. coli* genes, malate dehydrogenase (*mdh*) and phosphoenolpyruvate carboxylase (*ppc*), from multicopy plasmids. This strain produced 108 mM succinate with a molar yield of 0.98 mol succinate per mole of metabolized glucose (Chatterjee et al., 2001; Millard et al., 1996; Stols and Donnelly, 1997). Strain HL27659k was engineered by mutating succinate dehydrogenase (*sdhAB*), phosphotransacetylase (*pta*), acetate kinase (*ackA*), pyruvate oxidase (*poxB*), glucose transporter (*ptsG*), and the isocitrate lyase repressor (*iclR*). This strain produced less than 100 mM succinate and required oxygen-limited fermentation conditions (Cox et al., 2006; Lin et al., 2005a,b,c; Yun et al., 2005). Analysis of metabolism in silico

Correspondence to: L.O. Ingram

Contract grant sponsor: US Department of Energy
Contract grant number: FG02-96ER20222; FG36-04GO14019
Contract grant sponsor: BioEnergy International, LLC

Table I. Comparison of succinate production by microbial biocatalysts[a]

Organism	Medium/condition	Succinate titer (mM)[b]	Succinate yield (mol/mol)	Reference
E. coli KJ060 (*ldhA*, *adhE*, *ackA*, *focA*, *pflB*)	Glucose AM1 (100 g/L) with 10 g/L $NaHCO_3$, simple batch fermentation, 120 h incubation, pH maintained with 1:1 mixture of 6 M KOH + 3 M K_2CO_3	733 [0.90]	1.41	This article
E. coli KJ073 (*ldhA*, *adhE*, *ackA*, *focA*, *pflB*, *mgsA*, *poxB*)	Glucose AM1 (100 g/L) with 10 g/L $NaHCO_3$, simple batch fermentation, 96 h incubation, pH maintained with 1:1 mixture of 6 M KOH + 3 M K_2CO_3	668 [0.82]	1.20	This article
E. coli KJ060 (*ldhA*, *adhE*, *ackA*, *focA*, *pflB*) high inoculum (200 mg CDW L^{-1})	Glucose AM1 (100 g/L) with 10 g/L $NaHCO_3$, simple batch fermentation, 120 h incubation, pH maintained with 1:1 mixture of 6 M KOH + 3 M K_2CO_3	622 [0.61]	1.61	This article
Actinobacillus succinogenes FZ53	Glucose (130 g/L) supplemented with 15 g/L CSL and 5 g/L YE, 80 g/L $MgCO_3$, anaerobic batch fermentation, 78 h incubation	898 [1.36]	1.25	Guettler et al. (1996a)
E. coli AFP111 *(pflAB, ldhA, ptsG) Rhizobium etli pyc* overexpressed	Glucose (40 g/L; 90 g total glucose) in medium supplemented with 20 g/L tryptone, 10 g/L YE and 40 g/L $MgCO_3$, dual phase-fed batch fermentation, 76 h incubation	841 [1.31]	1.68	Vemuri et al. (2002a,b)
Anaerobiospirillum succiniciproducens ATCC 53488	Glucose (120 g/L) in peptone/YE-based medium, integrated membrane-bioreactor-electrodialysis with CO_2 sparging, 150 h incubation	703 [0.55]	1.35	Meynial-Salles et al. (2007)
A. succinogenes 130Z	Glucose (100 g/L) supplemented with 15 g/L CSL and YE, 80 g/L $MgCO_3$, anaerobic batch fermentation, CO_2 sparging, 39 h incubation	678 [2.05]	1.37	Guettler et al. (1996b)
E. coli HL27659k/pKK313 *(iclR, sdhAB, ackA-pta, poxB, pstG) Sorghum vulgare pepc* overexpressed	Glucose (106 g/L) in medium supplemented with 20 g/L tryptone, 32 g/L YE and 2 g/L $NaHCO_3$, fed batch fermentation under complete aerobic condition, 59 h incubation	499 [1.00]	0.85	Lin et al. (2005d)
A. succiniciproducens ATCC 53488	Glucose (50 g/L) and 10 g/L CSL, CO_2 sparging and 300 mM Na_2CO_3, batch fermentation, 24 h incubation	426 [2.09]	1.37	Glassner and Datta (1992)
Mannheimia succiniciproducens (ldhA, pflB, pta-ackA)	Glucose (63 g/L) in MMH3 (yeast extract based medium), fed batch fermentation, 0.25 vol/vol/min CO_2 sparging, 30 h incubation	444 [1.75]	1.16	Lee et al. (2006)
Bacterial isolate 130Z ATCC 55618	Glucose (50 g/L) supplemented with 1% CSL, 0.6% YE, and 2 g/L $MgCO_3$ neutralized with 10 N NaOH, 0.3 atm of CO_2, 29.5 h incubation	388 [1.55]	1.40	Guettler et al. (1998)
E. coli SBS550MG (*ldhA*, *adhE*, *iclR*, *ackA-pta*), *L. lactis pyc*, *Bacillus subtilis citZ*	Glucose (20 g/L; 100 g total glucose) LB supplemented with 1 g/L $NaHCO_3$, 200 mg/L ampicillin, and 1 mM IPTG. 100% CO_2 at 1 L/min STP headspace, repeated fed-batch fermentation, 95 h incubation	339 [0.42]	1.61[c]	Sanchez et al. (2005a), Cox et al. (2006)
E. coli AFP184 (*pflB*, *ldhA pts*)	Glucose (102 g/L) supplemented with 15 g/L CSL, dual phase aerobic growth and anaerobic production, sparging with air followed by CO_2, 32 h incubation	339 [1.27]	0.72[c]	Andersson et al. (2007)
A. succinogenes ATCC 55618	Glucose (70 g/L) with flour hydrolysate and 5 g/L YE, anaerobic batch fermentation with 4% inoculum, 65 h incubation	302 [0.55]	1.18	Du et al. (2007)
A. succiniciproducens ATCC 53488	Glucose (50 g/L), 2% CSL, and 25 ppm tryptophan, neutralized with 5.5 M $NaCO_3$, saturated medium of 0.3 atm partial pressure of CO_2, 29.5 h incubation	289 [1.16]	1.04	Guettler et al. (1998)
Succinivibrio dextrinosolvens ATCC 19716	CSL (15 g/L) and YE (15 g/L), 100 g/L glucose, and 80 g/L $MgCO_3$, batch fermentation, 36 h	226 [0.74]	NR	Guettler et al. (1998)
Corynebacterium glutanicum R	Glucose (40 g/L; 121 g total glucose) in defined mineral salt medium with 400 mM $NaHCO_3$, fed batch fermentation, 6 h incubation	195 [3.83]	0.29	Okino et al. (2005)

Table I. (*Continued*)

Organism	Medium/condition	Succinate titer (mM)[b]	Succinate yield (mol/mol)	Reference
Prevotella ruminocola ATCC 19188	CSL (15 g/L) and YE (15 g/L), 100 g/L glucose, and 80 g/L $MgCO_3$, batch fermentation, 36 h incubation	160 [0.52]	NR	Guettler et al. (1998)
E. coli SBS550MG (*ldhA*, *adhE*, *iclR*, *ackA-pta*), *L. lactis pyc*, *B. subtilis citZ*	Glucose LB (20 g/L) supplemented with 1 g/L $NaHCO_3$, 200 mg/L ampicillin, and 1 mM IPTG. 100% CO_2 at 1 L/min STP headspace, batch fermentation, 24 h incubation	162.6 [0.80]	1.61[c]	Sanchez et al. (2005a), Cox et al. (2006)
M. succiniciproducens MBEL55E KCTC 0769BP	Glucose (18 g/L) in MH4 (YE-based medium) supplemented with 119 mM $NaHCO_3$, a continuous-cell-recycle membrane reactor with the CO_2 partial pressure of 101.3 kPa gas (100% CO_2), 6 h incubation	144 [2.83]	1.44	Song et al. (2007)
E. coli SBS110MG *(ldhA*, *adhE)*, *Lactococcus lactis pyc*	Glucose LB (20 g/L) supplemented with1.5 g/L $NaHCO_3$ and 0.5 g $MgCO_3$, 200 mg/L ampicillin, and 1 mM IPTG. Dual phase with 100% CO_2 at 1 L/min STP headspace, 168 h incubation	130 [0.09]	1.24[c]	Sanchez et al. (2005a), Sanchez et al. (2006)
E. coli NZN111 (W1485 *pflB*, *ladhA*), *E. coli mdh* overexpressed	Glucose LB (20 g/L) supplemented with 0.5 g $MgCO_3$, 1.5 g/L NaOAc, 0.1 g/L ampicillin, and 10 μM IPTG, 44 h incubation, sealed serum tube	108 [0.22]	0.98[c]	Stols and Donnelly (1997)
E. coli JCL1208, *E. coli ppc* overexpressed	Glucose LB (11 g/L) supplemented with 0.15 g $MgCO_3$, 0.1 g/L carbenicillin, and 0.1 mM IPTG, 44 h incubation, anoxic CO_2 charging at 1 atm headspace, 18 h incubation	91 [0.60]	0.44[c]	Millard et al. (1996)
E. coli GJT–*Sorghum pepC*	Glucose LB (40 g/L) supplemented with 27.78 g/L $MgCO_3$, simple batch fermentation in sealed airtight flask	80 [no data]	0.42[c]	Lin et al. (2005c)
E. coli HL51276k *(iclR*, *icd*, *sdhAB*, *ackA-pta*, *poxB*, *pstG)*, *Sorghum sp. pepC* S8D mutation	Glucose LB (10.8 g/L) supplemented with 2 g/L $NaHCO_3$, 50 mg/L kanamycin, 1 mM IPTG, aerobic batch reactor, 50 h incubation	68 [0.16]	1.09[c]	Lin et al. (2005b)
E. coli SBS880MG (*ldhA*, *adhE*, Δ*fdhF*), *L. lactis pyc*	Glucose LB (20 g/L) supplemented with1.5 g/L $NaHCO_3$ and 0.5 g $MgCO_3$, 200 mg/L ampicillin, and 1 mM IPTG. Dual phase with 100% CO_2 headspace, 168 h incubation	60 [0.04]	0.94[c]	Sanchez et al. (2005b)

[a]Abbreviations: CSL, corn steep liquor; YE, yeast extract; NR, not reported.
[b]Average volumetric productivity is shown in brackets [g L^{-1} h^{-1}].
[c]The molar yield was calculated based on the production of succinate from metabolized sugar during both aerobic and anaerobic conditions. Biomass was generated predominantly during aerobic growth. Succinate was produced primarily during anaerobic incubation with CO_2, H_2, or a mixture of both.

has been used to design gene knockouts to create a pathway in *E. coli* that is analogous to the native succinate pathway in *M. succiniciproducens* (Lee et al., 2005, 2006). The resulting strain, however, produced low levels of succinate. Andersson et al. (2007) reported the highest levels of succinate production by an engineered *E. coli* (339 mM) containing only native genes.

Other researchers have pursued alternative approaches that express heterologous genes from plasmids in recombinant *E. coli* (Table I). The *Rhizobium etli* pyruvate carboxylase (*pyc*) was over-expressed from a multicopy plasmid to direct carbon flow into succinate (Gokarn et al., 2000; Vemuri et al., 2002a,b) and produced high levels of succinate (841 mM succinate). Strain SBS550MG was constructed by inactivating the isocitrate lyase repressor (*iclR*), *adhE*, *ldhA*, and *ackA*, and over-expressing *Bacillus subtilis citZ* (citrate synthase) and *R. etli pyc* from a multicopy plasmid (Sanchez et al., 2005a) to achieve a very high yield, 1.6 mol succinate/mol glucose.

Complex, multi-stage fermentation processes have also been investigated to improve succinate production using recombinant *E. coli* (Table I). In many of these, aerobic growth phase is followed by an anaerobic fermentation phase and includes sparging with CO_2, H_2, or both (Andersson et al., 2007; Nghiem et al., 1999; Sanchez et al., 2005a,b; Sanchez et al., 2006; Vemuri et al., 2002a,b). In a recent study with a native succinate producer, *A. succiniciproducens*, electrodialysis, sparging with CO_2, cell recycle, and batch feeding were combined (Meynial-Salles et al., 2007).

In this article, we describe novel strains of *E. coli* C that produce succinate at high titers and yields in mineral salts media during simple, pH-controlled, batch fermentations without the addition of heterologous genes or plasmids.

During development, an intermediate strain was characterized that produced malate as the dominant product.

Materials and Methods

Strains, Media, and Growth Conditions

Strains used in this study are summarized in Table II. Derivatives of *E. coli* C (ATCC 8739) were developed for succinate production by a unique combination of gene deletions and selections for increased productivity. Cultures were grown at 37°C in modified Luria–Bertani (LB) broth (per liter: 10 g Difco tryptone, 5 g Difco yeast extract, 5 g sodium chloride) (Miller, 1992) only during strain construction. Antibiotics were included as appropriate.

NBS mineral salts medium (Causey et al., 2004) supplemented with 100 mM $KHCO_3$, 1 mM betaine HCl, and sugar (2–10%) was used as a fermentation broth in most studies and for maintenance of strains. A new low salt

Table II. *Escherichia coli* strains, plasmids, and primers used in this study

Escherichia coli strains		
	Relevant characteristics	Sources
Strain C	Wild type (ATCC 8739)	ATCC
KJ012	strain C, Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT	This study
KJ017	KJ012, improved strain selected from 10% glucose, NBS	This study
KJ032	KJ017, Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT Δ(*focA-pflB*)::FRT	This study
KJ060	KJ032, improved strain selected from 10% glucose without initial acetate, NBS	This study
KJ070	KJ060, Δ*mgsA*	This study
KJ071	KJ070, improved strain selected from 10% glucose, NBS	This study
KJ072	KJ071, Δ*poxB*	This study
KJ073	KJ072, improved strain selected from 10% glucose, AM1	This study
SZ204	Δ(*focA-pflB*)::FRT-*kan*-FRT	Zhou et al. (2003)
Plasmids		
pKD4	bla FRT-*kan*-FRT	Datsenko and Wanner (2000)
pKD46	*bla γ β exo* (Red recombinase), temperature-conditional replicon	Datsenko and Wanner (2000)
pFT-A	*bla flp* temperature-conditional replicon and FLP recombinase	Posfai et al. (1997)
pEL04	*cat–sacB* targeting cassette	Lee et al. (2001), Thomason et al. (2005)
pLOI3421	1.8 kbp *Sma*I fragment containing *aac*	Wood et al. (2005)
pLOI4151	*bla cat; cat–sacB* cassette	This study
pCR2.1-TOPO	*bla kan*; TOPO TA cloning vector	Invitrogen
pLOI4228	*bla kan; yccT′-mgsA-helD′* (PCR) from *E.coli* C cloned into pCR2.1-TOPO vector	This study
pLOI4229	*cat–sacB* cassette PCR amplified from pLOI4151 (*Eco*RV digested) cloned into *mgsA* in pLOI4228	This study
pLOI4230	PCR fragment amplified from pLOI4228 (using *mgsA*-1/2 primers), kinase treated, then self-ligation	This study
pLOI4274	*bla kan*; *poxB* (PCR) from *E.coli* C cloned into pCR2.1-TOPO vector	This study
pLOI4275	*cat–sacB* cassette PCR amplified from pLOI4151 (*Eco*RV digested) cloned into *poxB* of pLOI4274	This study
pLOI4276	PCR fragment amplified from pLOI4274 (using *poxB*-1/2 primers), kinase treated, then self-ligation	This study
Primer sets		
ldhA	5′ATGAACTCGCCGTTTTATAGCACAAAACAGTACGACAAGAAGTACGTGTAGGCTGGAGCTGCTTC3′ 5′TTAAACCAGTTCGTTCGGGCAGGTTTCGCCTTTTTCCAGATTGCTCATATGAATATCCTCCTTAG3′	This study
adhE	5′ATGGCTGTTACTAATGTCGCTGAACTTAACGCACTCGTAGAGCGTGTGTAGGCTGGAGCTGCTTC3′ 5′TTAAGCGGATTTTTTCGCTTTTTTCTCAGCTTTAGCCGGAGCAGCCATATGAATATCCTCCTTAG3′	Zhou et al. (2003)
ackA	5′ATGTCGAGTAAGTTAGTACTGGTTCTGAACTGCGGTAGTTCTTCAGTGTAGGCTGGAGCTGCTTC3′ 5′TCAGGCAGTCAGGCGGCTCGCGTCTTGCGCGATAACCAGTTCTTCCATATGAATATCCTCCTTAG3′	Zhou et al. (2003)
focA-pflB	5′TTACTCCGTATTTGCATAAAAACCATGCGAGTTACGGGCCTATAAGTGTAGGCTGGAGCTGCTTC3′ 5′ATAGATTGAGTGAAGGTACGAGTAATAACGTCCTGCTGCTGTTCTCATATGAATATCCTCCTTAG3′	This study
JM*catsacB*	5′TTAGCTAGCATGTGACGGAAGATCACTTCG3′ 5′CCGCTAGCATCAAAGGGAAAACTGTCCATAT3′	This study
cat-up	5′AGAGAGGATATCTGTGACGGAAGATCACTTCG3′ 5′AGAGAGGATATCGAATTGATCCGGTGGATGAC3′	This study
mgsA-up/down	5′CAGCTCATCAACCAGGTCAA3′ 5′AAAAGCCGTCACGTTATTGG3′	This study
*mgsA*1/2	5′AGCGTTATCTCGCGGACCGT3′ 5′AAGTGCGAGTCGTCAGTTCC3′	This study
poxB-up/down	5′AAGCAATAACGTTCCGGTTG3′ 5′CCACTTTATCCAGCGGTAGC3′	This study
poxB-1/2	5′GACGCGGTGATGAAGTGAT3′ 5′TTTGGCGATATAAGCTGCAA3′	This study

medium, AM1 (4.2 g L^{-1} total salts; Martinez et al., 2007), was developed during the latter stages of this investigation and used in fermentations with KJ060 and KJ073. This medium was supplemented with 100 mM $KHCO_3$ and sugar as indicated and includes 1 mM betaine when initial sugar concentrations are 5% or higher. No genes encoding antibiotic resistance, plasmids, or other foreign genes are present in strains developed for succinate production except as intermediates during construction.

Genetic Methods

Plasmids and primers used in this study are summarized in Table II. Methods for chromosomal deletions, integration, and removal of antibiotic resistance genes have been previously described (Datsenko and Wanner, 2000; Grabar et al., 2006; Posfai et al., 1997; Zhou et al., 2006a). Sense primers contain sequences corresponding to the N-terminus of each targeted gene (boldface type) followed by 20 bp (underlined) corresponding to the FRT-*kan*-FRT cassette. Anti-sense primers contain sequences corresponding to the C-terminal region of each targeted gene (boldface type) followed by 20 bp (underlined) corresponding to the cassette. Amplified DNA fragments were electroporated into *E. coli* strains harboring *Red* recombinase (pKD46). In resulting recombinants, the FRT-*kan*-FRT cassette replaced the deleted region of the target gene by homologous recombination (double-crossover event). The resistance gene (FRT-*kan*-FRT) was subsequently excised from the chromosome with FLP recombinase using plasmid pFT-A, leaving a scar region containing one FRT site. Chromosomal deletions and integrations were verified by testing for antibiotic markers, PCR analysis, and analysis of fermentation products. Generalized P1 phage transduction (Miller, 1992) was used to transfer the Δ*focA-pflB*::FRT-*kan*-FRT mutation from strain SZ204 into strain KJ017 to produce KJ032.

Deletion of *mgsA* and *poxB* Genes

A modified method was developed to delete *E. coli* chromosomal genes using a two-step homologous recombination process (Thomason et al., 2005). With this method, no antibiotic genes or scar sequences remain on the chromosome after gene deletion. In the first recombination, part of the target gene was replaced by a DNA cassette containing a chloramphenicol resistance gene (*cat*) and a levansucrase gene (*sacB*). In the second recombination, the *cat–sacB* cassette was replaced with native sequences omitting the region of deletion. Cells containing the *sacB* gene accumulate levan during incubation with sucrose and are killed. Surviving recombinants are highly enriched for loss of the *cat–sacB* cassette.

A cassette was constructed to facilitate gene deletions. The *cat–sacB* region was amplified from pEL04 (Lee et al., 2001; Thomason et al., 2005) by PCR using the JM*catsacB* primer set (Table II), digested with *Nhe*I, and ligated into the corresponding site of pLOI3421 to produce pLOI4151. The *cat–sacB* cassette was amplified by PCR using pLOI4151 (template) and the *cat*-up primer set (*EcoRV* site included in each primer), digested with *EcoRV*, and used in subsequent ligations.

The *mgsA* gene and neighboring 500 bp regions (*yccT′-mgsA-helD′*, 1,435 bp) were amplified using primer set *mgsA*-up/down and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) to produce plasmid pLOI4228. A 1,000-fold diluted preparation of this plasmid DNA served as a template for inside-out amplification using the *mgsA*-1/2 primer set (both primers within the *mgsA* gene and facing outward). The resulting 4,958 bp fragment containing the replicon was ligated to the amplified, *EcoRV*-digested *cat–sacB* cassette from pLOI4151 to produce pLOI4229. This 4,958 bp fragment was also used to construct a second plasmid, pLOI4230 (phosphorylation and self-ligation). In pLOI4230, the central region of *mgsA* is absent (*yccT′-mgsA′-mgsA″-helD′*).

After digestion of pLOI4229 and pLOI4230 with *Xmn*I (within the vector), each served as a template for amplification using the *mgsA*-up/down primer set to produce the linear DNA fragments for integration step I (*yccT′-mgsA′-cat-sacB-mgsA″-helD′*) and step II (*yccT′-mgsA′-mgsA″-helD′*), respectively. After electroporation of the step I fragment into KJ060 containing pKD46 (Red recombinase) and 2 h of incubation at 30°C to allow expression and segregation, recombinants were selected for chloramphenicol (40 mg L^{-1}) and ampicillin (20 mg L^{-1}) resistance on plates (30°C, 18 h). Three clones were chosen, grown in Luria broth with ampicillin and 5% w/v arabinose, and prepared for electroporation. After electroporation with the step II fragment, cells were incubated at 37°C for 4 h and transferred into a 250-mL flask containing 100 mL of modified LB (100 mM MOPS buffer added and NaCl omitted) containing 10% sucrose. After overnight incubation (37°C), clones were selected on modified LB plates (no NaCl; 100 mM MOPS added) containing 6% sucrose (39°C, 16 h). Resulting clones were tested for loss of ampicillin and chloramphenicol resistance. Construction was further confirmed by PCR analysis. A clone lacking the *mgsA* gene was selected and designated KJ070.

The *poxB* gene was deleted from KJ071 in a manner analogous to that used to delete the *mgsA* gene. Additional primer sets (*poxB*-up/down and *poxB*-1/2) used to construct the *poxB* deletion are included in Table II together with the corresponding plasmids (pLOI4274, pLOI4275, and pLOI4276). The resulting strain was designated KJ072.

Fermentations

Seed cultures and fermentations were grown at 37°C, 100 rpm in NBS or AM1 mineral salts medium containing glucose, 100 mM $KHCO_3$ and 1 mM betaine HCl. These were maintained at pH 7.0 by the automatic addition of KOH during initial experiments. Subsequently, pH was maintained by adding a 1:1 mixture of 3 M K_2CO_3 and 6 N KOH. Fermentations were carried out in small fermentation vessels

with a working volume of 350 mL. Fermentations were inoculated at either an initial OD_{550} of 0.01 (3.3 mg CDW L^{-1}) or 0.1 (33.3 mg CDW L^{-1}) as indicated. No antibiotic resistance genes were present in the strains that were tested. Fermentation vessels were sealed except for a 16-gauge needle, which served as a vent for sample removal. Anaerobiosis was rapidly achieved during growth with added bicarbonate serving to ensure an atmosphere of CO_2.

Analyses

Cell mass was estimated from the optical density at 550 nm (OD 1.0 = 333 mg of cell dry weight L^{-1}) with a Bausch & Lomb Spectronic 70 spectrophotometer. Organic acids and sugars were determined by using high performance liquid chromatography (Grabar et al., 2006).

Results and Discussion

Construction of KJ012 for Succinate Production by Deletion of *ldhA*, *adhE*, and *ackA*

E. coli produces a mixture of lactate, acetate, ethanol, and succinate during glucose fermentation (Fig. 1A). Major pathways leading to lactate, acetate, and ethanol were eliminated by deleting genes encoding D-lactate dehydrogenase, acetate kinase, and alcohol dehydrogenase to construct KJ012(Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT).

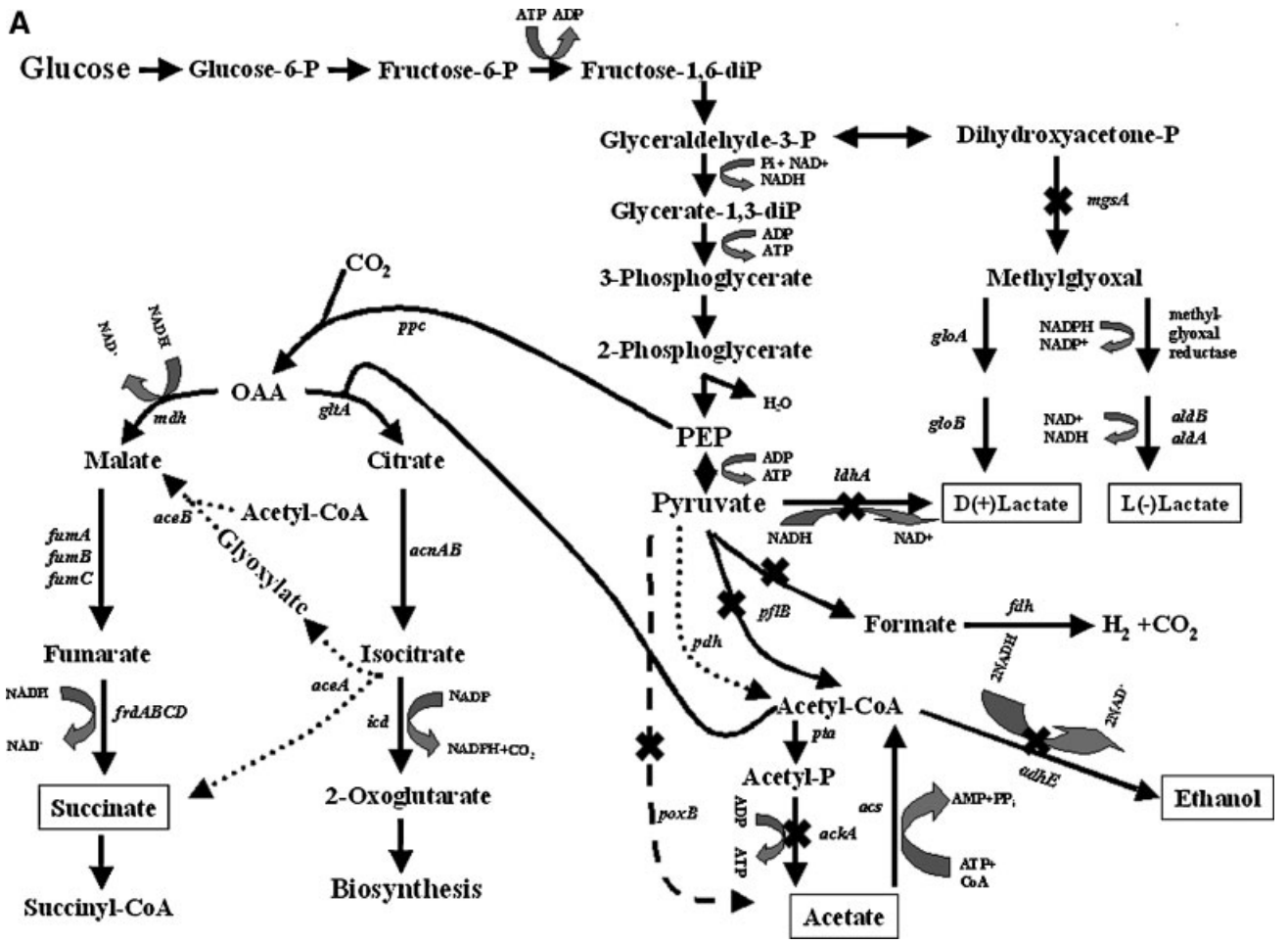

Figure 1. Fermentation of glucose to succinate. **A**: Central metabolism indicating genes deleted in constructs engineered for succinate production. Solid arrows represent central fermentative pathways. Dashed arrow represents microaerophilic pathway (*poxB*). Dotted arrows show pathways that normally function during aerobic metabolism, pyruvate dehydrogenase (*pdh*) and the glyoxylate bypass (*aceAB*). Crosses represent the gene deletions performed in this study to obtain KJ012 (*ldhA, adhE, ackA*), KJ032 (*ldhA, adhE, ackA, focA, pflB*), and KJ070 (*ldhA, adhE, ackA, pflB, mgsA*), and KJ072 (*ldhA, adhE, ackA, focA, pflB, mgsA, poxB*). Genes and enzymes: *ldhA*, lactate dehydrogenase; *focA*, formate transporter; *pflB*, pyruvate-formate lyase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *adhE*, alcohol dehydrogenase; *ppc*, phosphoenolpyruvate carboxylase; *pdh*, pyruvate dehydrogenase complex; *gltA*, citrate synthase; *mdh*, malate dehydrogenase; *fumA*, *fumB*, and *fumC*, fumarase isozymes; *frdABCD*, fumarate reductase; *fdh*, formate dehydrogenase; *mgsA*, methylglyoxal synthase; *gloAB*, glyoxylase I and II; *poxB*, pyruvate oxidase; *aceAB*, isocitrate lyase; *acnAB*, aconitase; *acs*, acetyl-CoA synthase, *aldA* and *aldB*, aldehyde dehydrogenase isozymes; and *icd*, isocitrate dehydrogenase. **B**: Coupling of ATP production and growth to succinate and malate production in engineered strains of *E. coli*. Solid arrows connect NADH pools. Dotted arrows connect NAD^+ pools. During glycolysis under anaerobic conditions, the production of ATP for cell growth is obligately coupled to the oxidation of NADH.

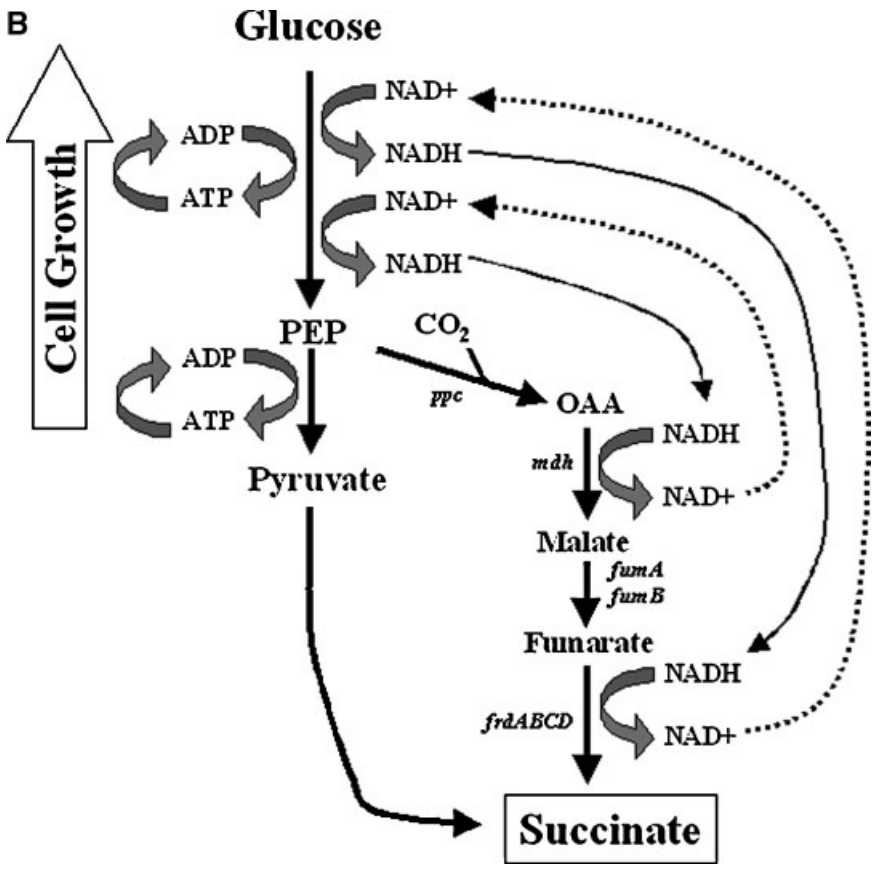

Figure 1. (*Continued*)

This strain retained only the succinate pathway with malate dehydrogenase and fumarate reductase as primary routes for NADH oxidation (Fig. 1B). Although strain KJ012 grew well in complex media (not shown), poor growth was observed in mineral-based media such as NBS and acetate was produced as the most abundant product from sugar metabolism (Table III). In this NBS mineral salts medium, succinate titer and cell yield for KJ012 were eightfold to sevenfold lower than the unmodified parent, *E. coli* C.

Poor growth and glucose fermentation could result from insufficient capacity to oxidize NADH using only the succinate pathway (Fig. 1A), from a deficiency of metabolites and precursors for biosynthesis (error in metabolic partitioning) due to shifts in pool sizes resulting from gene deletions, or a combination of both. Growth was increased fivefold (Table III) in the same medium (plus 100 mM MOPS for pH control) by providing mild aeration (100 rpm, 100 mL NBS broth, 250-mL flask) indicating that the NADH oxidation capacity limited growth. Growth was also increased (fivefold) by replacing NBS mineral salts medium with Luria broth indicating a deficiency in metabolic partitioning. With the complex nutrients of Luria broth, succinate titers were increased 18-fold over NBS medium indicating that the succinate pathway served as the primary route for NADH oxidation in KJ012. From these results, we concluded that the poor performance of KJ012 under anaerobic conditions is a complex problem resulting primarily from suboptimal metabolic partitioning of carbon between growth and fermentation products.

It is likely that similar observations have been made by previous investigators with *E. coli* engineered for succinate production and that this is the basis for their reliance on complex medium and two-phase process (aerobic growth and anaerobic production). In the absence of a clear approach to decrease the complexity of this problem in engineered *E. coli*, we elected to pursue an adaptive genetic strategy termed ''metabolic evolution.'' Rather than incremental studies to identify and solve specific problems in metabolic partitioning, natural selection was used to solve the aggregate problem. The production of ATP for growth by KJ012 and all subsequent derivatives in this article is obligately coupled to malate and succinate production during the oxidation of NADH (Fig. 1B). Selection for improved growth and ATP production during serial cultivation provides a route to co-select improved growth and improved production of these dicarboxylic acids. Analogous

Table III. Fermentation of glucose in mineral salts medium by mutant strains of *E. coli*

Strain[a]	Culture conditions	Media, gluc (w/v)	Cell yield[b] (g/L)	Succinate yield[c]		Average volumetric productivity[d] (g/L/h)	Fermentation products (mM)[e]					
				mol/mol	g/g		Suc	Mal	Pyr	Ace	Lac	For
E. coli C wild type[f]	OD_{550}, 0.1 mM betaine	5%, NBS	2.0 ± 0.2	0.19 ± 0.02	0.12	0.12 ± 0.01	49 ± 3	—[g]	33 ± 10	152 ± 30	98 ± 24	262 ± 19
KJ012[f]	0.1 OD_{550}, 0.1 mM betaine	5% NBS	0.3 ± 0.1	0.20 ± 0.01	0.13	0.04 ± 0.01	6 ± 0.4	—	—	26 ± 1	—	—
KJ012	0.1 OD_{550}, 0.1 mM betaine shaken flask[h]	5% NBS + MOPS	1.5	0.10	0.06	0.02	10	—	—	226	—	16
KJ012	0.1 OD_{550} Luria Broth	5% LB	1.5	0.70	0.50	0.09	108	—	—	61	<2	14
KJ012 (*ldhA*, *ackA*, *adhE*) (KJ017)	1st TF: No betaine, 0.1 OD_{550}, 120 h transfers	5%, NBS	0.3	0.13	0.09	0.072	6	—	—	26	<2	—
	3rd TF: 2 mM betaine, 0.1 OD_{550}, 96 h transfers	5%, NBS	0.7	0.28	0.18	0.128	26	—	—	71	<2	—
	40th TF: 1 mM betaine, 0.1 OD_{550}, 24 h transfers, 3 M K_2CO_3 + 6 N KOH	5%, NBS	2.3	0.73	0.48	0.251	204	—	—	179	<2	151
	40th TF: 1 mM betaine, 0.1 OD_{550}, 24 h transfers, 3 M K_2CO_3 + 6 N KOH	10%, NBS	1.7	0.74	0.49	0.354	288	—	—	181	38	199
KJ032 (*ldhA*, *ackA*, *adhE*, *focA*, *pflB*) (KJ060)	2nd TF: 1 mM betaine, 0.1 OD_{550}, 48 h transfers, 20 mM NaOAc, 3 M K_2CO_3 + 6 N KOH	5%, NBS	1.0	1.47	0.97	0.260	212	—	—	44	—	—
	15th TF: 1 mM betaine, 0.01 OD_{550}, 24 h transfers, 5 mM NaOAc, 3 M K_2CO_3 + 6 N KOH	10%, NBS	1.4	1.07	0.71	0.736	596	331	9	170	<2	—
	5th TF: 1 mM betaine, 0.01 OD_{550}, 24 h transfers, No NaOAc, 3 M K_2CO_3 + 6 N KOH	10%, NBS	1.4	1.04	0.69	0.711	579	318	9	161	<2	—
KJ070 (*ldhA*, *ackA*, *adhE*, *focA*, *pflB*, *mgsA*) (KJ071)	1st TF: 1 mM betaine, 0.01 OD_{550}, 24 h TF, 3 M K_2CO_3 + 6 N KOH	5%, NBS	1.0	1.06	0.70	0.361	294	219	25	102	—	—
	50th TF: 1 mM betaine, 0.01 OD_{550}, 24 h transfers, 3 M K_2CO_3 + 6 N KOH	10%, NBS	1.1	0.71	0.47	0.419	341	626	<2	76	—	—
KJ072 (*ldhA*, *ackA*, *adhE*, *focA pflB*, *mgsA*, *poxB*) (KJ073)	2nd TF: 1 mM betaine, 0.01 OD_{550}, 24 h transfers, 3 M K_2CO_3 + 6 N KOH	10%, NBS	1.3	0.97	0.64	0.663	539	186	<2	95	—	—
	6th TF: 1 mM betaine, 0.01 OD_{550}, 24 h transfers, 3 M K_2CO_3 + 6 N KOH	10%, AM1	1.2	1.34	0.88	0.733	596	38	4	112	—	—
	45th TF: 1 mM betaine, 0.01 OD_{550}, 24 h transfers, 3 M K_2CO_3 + 6 N KOH	10%, AM1	1.5	1.26	0.83	0.858	699	313	103	172	—	—
KJ073[f]	1 mM betaine, 3 M K_2CO_3 + 6 N KOH 0.01 OD_{550} inoculum	10%, AM1	2.3 ± 0.1	1.20 ± 0.09	0.77 ± 0.03	0.82 ± 0.01	668 ± 8	118 ± 13	55 ± 22	183 ± 27	—	—
KJ060[f]	1 mM betaine, 3 M K_2CO_3 + 6 N KOH 0.01 OD_{550} inoculum	10%, AM1	2.2 ± 0.1	1.41 ± 0.07	0.92 ± 0.05	0.90 ± 0.04	733 ± 39	39 ± 17	—	250 ± 36	2 ± 1	—
KJ060[f]	1 mM betaine, 3 M K_2CO_3 + 6 N KOH 0.60 OD_{550} inoculum	10%, AM1	2.2 ± 0.1	1.61 ± 0.12	1.05 ± 0.09	0.77 ± 0.04	622 ± 8	17 ± 5	1.5 ± 1	180 ± 13	2 ± 1	—
KJ071[f]	1 mM betaine, 3 M K_2CO_3 + 6 N KOH 0.01 OD_{550} inoculum	10%, NBS	1.5 ± 0.0	0.78 ± 0.02	0.53 ± 0.01	0.33 ± 0.04	280 ± 7	516 ± 14	58 ± 15	64 ± 9	—	—

[a]Clones were isolated from the fermentation broth at various points and assigned strain numbers, indicated by numbers in parentheses.
[b]Cell yield estimated from optical density (3 $OD_{550\ nm}$ = 1 g L^{-1} CDW).
[c]Succinate yields were calculated based on glucose metabolized.
[d]Average volumetric productivity was calculated for total incubation time.
[e]Abbreviations: suc, succinate; mal, malate; pyr, pyruvate; ace, acetate; lac, lacate; for, formate.
[f]Average of three or more fermentations with standard deviations.
[g]Dash indicates absence of product.
[h]Aerobic shaken flask (100 rpm; 100 mL NBS, 250-mL flask).

approaches have been used to develop *E. coli* strains for the production of L-alanine (Zhang et al., 2007) and lactate (Zhou et al., 2003; Zhou et al., 2006a,b).

Improvement of KJ012 by Metabolic Evolution

Metabolic evolution was carried out by sequentially subculturing under various regimens using small, pH-controlled fermentors (Fig. 2). Selection was begun using 5% (w/v) glucose with serial transfers at 120-h intervals (Figs. 3A and 4A). Addition of betaine, a protective osmolyte (Purvis et al., 2005; Underwood et al., 2004; Zhou et al., 2006b), increased cell growth and cell yield and allowed more frequent transfers. Beginning at transfer 27, a bicarbonate solution was used to maintain pH and provide additional CO_2 for succinate production. The rapid subsequent improvement confirmed that CO_2 had become limiting (Fig. 4A). With further selection in 5% (w/v) glucose, molar yields of succinate improved to 0.73 per mole of glucose metabolized (Table III). The glucose concentration was doubled and transfers continued (Figs. 3B and 4B) with modest improvement in growth (initial 24 h) and a small decline in succinate yield. With 10% (w/v) glucose, unwanted co-products (acetate, formate, and lactate) were abundant despite the absence of the primary lactate dehydrogenase (*ldhA*) and acetate kinase (*ackA*) (Table III). A clone was isolated from the last transfer and designated KJ017(Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT).

Construction of KJ032 and KJ060

The gene encoding pyruvate formate-lyase (*pflB*) was deleted from KJ017 to eliminate the loss of reductant as formate and excessive production of acetyl-CoA, a potential source of acetate. The upstream formate transporter (*focA*) in this operon was also deleted. As expected, this deleted strain (KJ032) did not grow without acetate (Fig. 3C). Deletion of *pflB* is well-known to cause acetate auxotrophy under anaerobic conditions (Sawers and Bock, 1988). Growth and succinate production by KJ032 were restored by the addition of 20 mM acetate (Figs. 3C and 4C). Production of formate and acetate were substantially reduced as a result of *pflB* (and *focA*) deletion. Although this strain required acetate for growth, additional acetate was also produced during fermentation. The same phenomenon was previously reported for *pflB*-deleted strains during the construction of *E. coli* K-12 biocatalysts for pyruvate production (Causey et al., 2004). Lactate levels were also reduced in KJ032

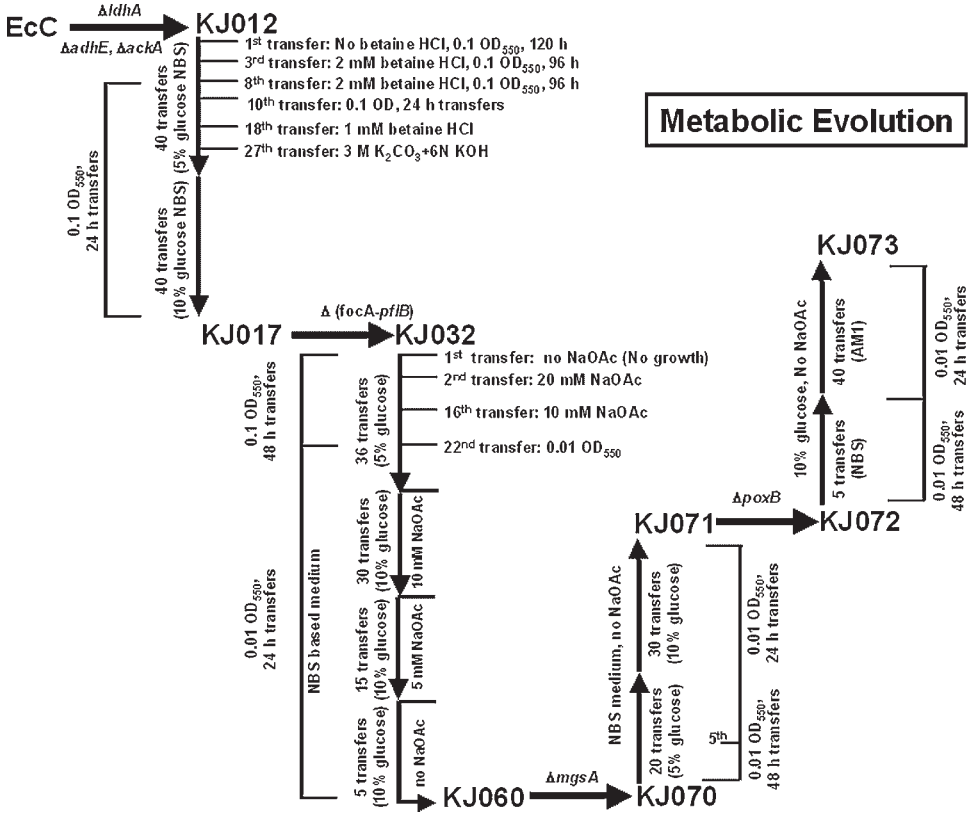

Figure 2. Diagram summarizing steps in the genetic engineering and metabolic evolution of *E. coli* C as a biocatalyst for succinate and malate production. This process represents 261 serial transfers providing over 2,000 generations of growth-based selection. Clones were isolated from the final culture of each regimen and assigned strain designations, shown in parentheses in Table III.

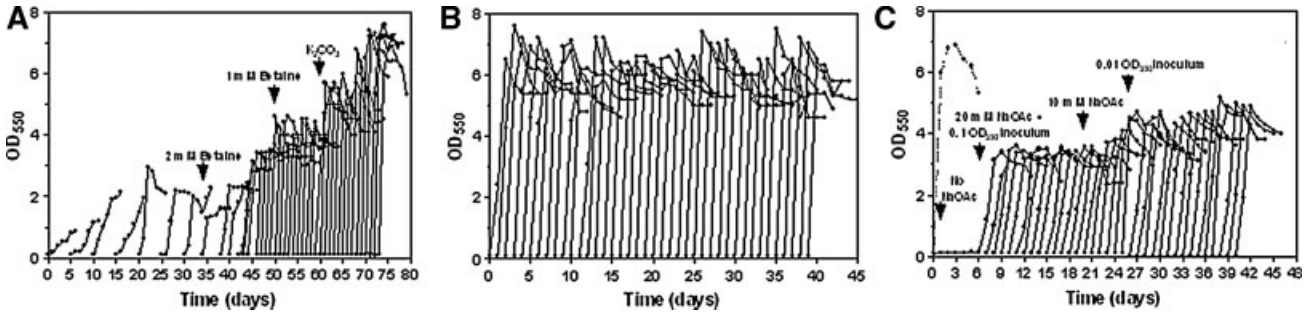

Figure 3. Growth during metabolic evolution of KJ012 to produce KJ017, KJ032, and KJ060. Strain KJ012 was sequentially transferred in NBS medium containing 5% (w/v) (**A**) and 10% (w/v) (**B**) glucose, respectively to produce KJ017. After deletion of *focA* and *pflB*, the resulting strain (KJ032) was initially subcultured in medium supplemented with acetate (**C**). Acetate levels were decreased and subsequently eliminated during further transfers to produce KJ060. Broken line represents fermentation by KJ017 without acetate, added for comparison. Symbols: optical density at $OD_{550\ nm}$, ●.

(Table III; Fig. 4C). Subsequent transfers were accompanied by improvements in growth and succinate production. Added acetate was reduced, inocula size was reduced, and glucose concentration was doubled (10% w/v) during subsequent transfers (Fig. 4D). After reducing added acetate to 5 mM, an unstable population emerged that produced elevated levels of malate at the expense of succinate. After further transfers, acetate was omitted and a strain was developed that was no longer auxotrophic. However, succinate yields declined upon elimination of added acetate while malate and acetate levels increased. A small amount of pyruvate was also produced. A clone was isolated from the last transfer and designated, KJ060(Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT Δ*focB-pflB*::FRT).

Construction of KJ070 and KJ071 by Deletion of Methylglyoxal Synthase (*mgsA*)

The small amount of lactate present in the fermentation broths of various strains is presumed to originate from the methylglyoxal synthase pathway (Fig. 1A; Grabar et al., 2006). Although this represents a small loss of yield, lactate production by this pathway is indicative of methylglyoxal

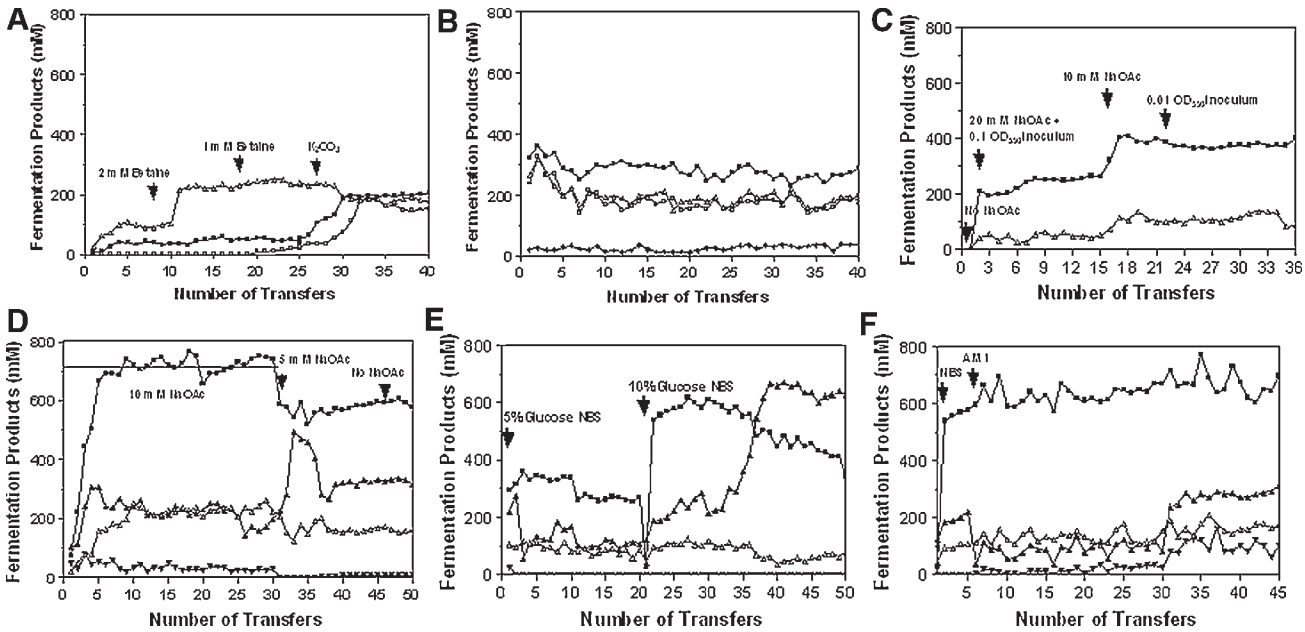

Figure 4. Summary of fermentation products during the metabolic evolution of strains for succinate and malate production. Cultures were supplemented with sodium acetate as indicated. Black arrows represent the transition between fermentation conditions as indicated by text. No formate and only small amounts of lactate were detected during metabolic evolution of KJ032. No formate and lactate were detected during metabolic evolution of KJ070 and KJ072. **A**: (5% w/v glucose) and (**B**) (10% w/v glucose), KJ012 to KJ017; (**C**) (5% w/v glucose) and (**D**) (10% w/v glucose), KJ032 to KJ060; (**E**) 10% glucose, KJ070 to KJ071; (**F**) 10% glucose, KJ072 to KJ073. Symbols for all: ■, succinate; □, formate; Δ, acetate; ▲, malate; ◆,lactate; and ▼, pyruvate.

accumulation, an inhibitor of both growth and glycolysis (Egyud and Szent-Gyorgyi, 1966; Grabar et al., 2006; Hopper and Cooper, 1971). Production of methylglyoxal and lactate were eliminated by deleting the *mgsA* gene (methylglyoxal synthase) in KJ060 to produce KJ070(Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT Δ*focA-pflB*::FRT Δ*mgsA*). Strain KJ070 was initially subcultured in 5% (w/v) glucose (Fig. 4E). Deletion of *mgsA* is presumed to have increased glycolytic flux as evidenced by the accumulation of pyruvate in the medium (Table III). This increase in glycolytic flux may also be responsible for the further decline in the succinate/malate ratio due to increased production of oxaloacetate, an allosteric inhibitor of fumarate reductase (Iverson et al., 2002; Sanwal, 1970).

At transfer 21, glucose was doubled to 10% (w/v) and transfers continued. This higher level of glucose and subsequent transfers resulted in further increases in malate production, exceeding succinate in latter transfers (Fig. 4E). Increased production of malate versus succinate in 10% w/v glucose is also consistent with increased glycolytic flux and inhibition of furmarate reductase by oxaloacetate. At transfer 50, 1.3 moles of malate and 0.71 moles of succinate were produced per mole of glucose metabolized (Table III). Significant amounts of acetate were also produced. A new strain was isolated from the final subculture and designated KJ071(Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT Δ*focA-pflB*::FRT Δ*mgsA*). This strain may be useful for malate production.

Construction of KJ072 and KJ073 by Deletion of *poxB*

Although conversion of glucose to acetate is redox neutral, partitioning of carbon to acetate decreases the yield of succinate and malate. Pyruvate oxidase represents a potential source of acetate and CO_2 during incubation under microaerophilic conditions (Causey et al., 2004) and was targeted for gene deletion. However, deletion of *poxB* to produce KJ072(Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT Δ*focA-pflB*::FRT Δ*mgsA* Δ*poxB*) did not reduce acetate production indicating that alternative pathways are involved. Eliminating *poxB* resulted in unexpected changes in fermentation products, an increase in succinate and decrease in malate (Table III; Fig. 4F). The mechanism for this improvement in succinate production is unknown but may be related to other activities of pyruvate oxidase such as acetoin production, decarboxylation, and carboligation (Ajl and Werkman, 1948; Chang and Cronan, 2000).

Strain KJ072 was subjected to 40 further rounds of metabolic evolution in AM1 medium, a lower salt medium, with 10% (w/v) glucose (Table III; Fig. 4F). Improvements in growth, cell yield, and succinate production were observed during these transfers. Malate, pyruvate, and acetate levels also increased. A clone was isolated from the final transfer and designated KJ073(Δ*ldhA*::FRT Δ*adhE*::FRT Δ*ackA*::FRT Δ*pflB*::FRT Δ*mgsA* Δ*poxB*).

Fermentation of KJ060 and KJ073 in AM1 Medium Containing 10% (w/v) Glucose

Figure 5 shows batch fermentations with KJ060 and KJ073, the two best biocatalysts for succinate production. Although growth was completed within the initial 48 h of incubation, succinate production continued for 96 h. One-third of succinate production occurred in the absence of cell growth. These strains produced succinate titers of 668–733 mM, with a molar yield of 1.2–1.6 based on glucose metabolized. Acetate, malate, and pyruvate accumulated as undesirable co-products and detracted from the potential yield of succinate (Table III). The maximum theoretical yield of succinate from glucose and CO_2 (excess) is 1.71 mol per mole glucose based on the following equation:

$$7\ C_6H_{12}O_6 + 6\ CO_2 \rightarrow 12\ C_4H_6O_4 + 6\ H_2O$$

Conversion of Other Substrates to Succinate

Although this study primarily focused on the conversion of glucose to succinate. It is well known that *E. coli* has the native ability to metabolize all hexose and pentose sugars

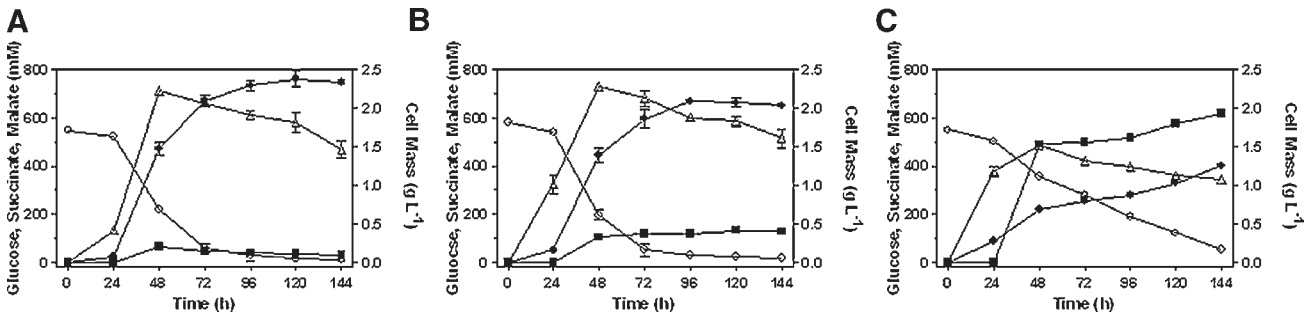

Figure 5. Production of succinate and malate in mineral salts media (10% glucose) by derivatives of *E. coli* C. **A**: Succinate production by KJ060 in AM1 medium. **B**: Succinate production by KJ073 in AM1 medium. **C**: Production of malate by KJ071 in NBS medium. Fermentations were inoculated at a level of 33 mg DCW L^{-1}. Symbols for all: ○, glucose; ●, succinate; ■, malate; Δ, cell mass.

that are constituents of plant cell walls (Asghari et al., 1996; Underwood et al., 2004). Some strains of *E. coli* can also metabolize sucrose (Moniruzzaman et al., 1997). Strain KJ073 was tested for utilization of hexoses, pentoses, and using tube cultures containing 2% sugar. In all cases, these sugars were converted primarily to succinate. Strain KJ073 also metabolized glycerol to succinate. During incubation with 2% glycerol, 143 mM glycerol was metabolized to produce 127 mM succinate with a molar yield of 0.89, 89% of the theoretical maximum.

Production of Malate in NBS Medium Containing 1 mM Betaine and 10% Glucose

During growth-based selections, cultures were observed to vary in their production of malate (Table III), a potentially useful alternative product. Malate was the most abundant product from KJ071 with 10% glucose (Table III; Fig. 4E), almost double that of succinate. This strain produced 516 mM malate with a molar yield of 1.44 based on metabolized glucose.

Conclusions

The fermentative metabolism of *E. coli* is remarkably adaptable. Derivatives can be readily engineered and evolved to circumvent numerous deletions of genes concerned with native fementation pathways and increase fluxes through remaining enzymes to maintain redox balance, ATP production, and growth. Though arguably more challenging, cells can make such adaptive changes in mineral salts media while balancing carbon partitioning for biosynthetic needs. After eliminating the primary routes for NADH oxidation (lactate dehydrogenase, alcohol dehydrogenase) and acetate production (acetate kinase), growth and ATP production remain linked to NADH oxidation and the production of malate or succinate (Fig. 1B). Growth-based selections for NADH oxidation and ATP production cannot readily distinguish between malate and succinate as end products, since the precursors of both serve as electron acceptors. During these investigations, KJ071 was developed that produces more malate than succinate. This strain and further derivatives may be useful for malate production. Other strains such as KJ073 and KJ060 may be useful for succinate production.

Deletion of *pflB*, the primary source of acetyl-CoA during anerobic growth, resulted in an auxotrophic requirement for acetate (Sawers and Bock, 1988). This requirement was eliminated through metabolic evolution, presumably due to increased production of acetyl-CoA by other routes such as pyruvate dehydrogenase (de Graef et al., 1999). Many shifts in metabolic products were unanticipated. The increase in malate during selections after deletion of *mgsA* is unexplained. Methylglyoxal is a metabolic inhibitor that is produced in response to an imbalance in metabolism (Grabar et al., 2006). Elimination of methylglyoxal production may have provided a growth-related advantage such as increased growth rate, a shorter lag after inoculation, etc. The reduction in malate and shift to higher succinate production after a *poxB* deletion was also surprising. Little change in the acetate level was observed indicating that either this enzyme was a minor source of acetate or that it was functionally replaced by other routes for acetate production. After deletion of *poxB*, succinate was again produced as the dominant dicarboxylic acid. With the best strains for succinate production, KJ060 and KJ073, malate and acetate remained as abundant co-products (Table III; Fig. 4D and F). Elimination of these represents a further opportunity to increase yields.

All previously engineered *E. coli* developed for succinate production have used complex media and plasmids with antibiotics for maintenance. Most have achieved only low titers of succinate in simple batch fermentations, requiring more complex processes to achieve higher titers (Table I). A variety of genetic approaches have been reported that increase succinate production from glucose by recombinant *E. coli* in complex medium. In our initial construct, growth and sugar metabolism were very poor in mineral salts medium but very robust in complex, LB medium. It is likely that similar observations led previous investigators to their reliance on complex nutrients (Table I). Complex media containing vitamins, amino acids, and other macromolecular precursors may mask potential regulatory problems in metabolism and biosynthesis that were created by metabolic engineering.

Many investigators have also used heterologous genes and complicated processes that include sparging with gas (CO_2, H_2, O_2, or air) and dual aerobic and anaerobic process steps. This complexity of process and nutrients would be expected to increase the cost of construction, materials, purification, and waste disposal. In contrast, strains KJ060 and KJ073 produced high titers of succinate (600–700 mM) in simple batch fermentations (10% sugar) using mineral salts medium without any complex nutrients or foreign genes.

The authors thank Dr. Karp and colleagues for continuing improvements in the EcoCyc database (Keseler et al., 2005), a tool used extensively throughout this work. The authors also thank Lorraine Yomano and Sean York for their instruction in molecular cloning and fermentation. This research was supported by grants from the US Department of Energy (FG02-96ER20222 and FG36-04GO14019), BioEnergy International, LLC, and K. Jantama was supported by a Royal Thai Government Scholarship.

References

Ajl SJ, Werkman CH. 1948. Enzymatic fixation of carbon dioxide in α-ketoglutaric acid. Proc Natl Acad Sci USA 34:491–498.

Andersson C, Hodge D, Berglund KA, Rova U. 2007. Effect of different carbon sources on the production of succinic acid using metabolically engineered *Escherichia coli*. Biotechnol Prog 23(2):381–388.

Asghari A, Bothast RJ, Doran JB, Ingram LO. 1996. Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* strain KO11. J Ind Microbiol 16:42–47.

Causey TB, Shanmugam KT, Yomano LP, Ingram LO. 2004. Engineering *Escherichia coli* for efficient conversion of glucose to pyruvate. Proc Natl Acad Sci USA 101:2235–2240.

Chang YY, Cronan JE, Jr. 2000. Conversion of *Escherichia coli* pyruvate decarboxylase to an 'alpha-ketobutyrate oxidase'. Biochem J 352:717–724.

Chatterjee R, Cynthia SM, Kathleen C, David PC, Donnelly MI. 2001. Mutation of the *ptsG* gene results in increased production of succinate in fermentation of glucose by *Escherichia coli*. Appl Environ Microbiol 67:148–154.

Cox SJ, Shalel LS, Sanchez AM, Lin H, Peercy B, Bennett GN, San KY. 2006. Development of a metabolic network design and optimization framework incorporating implement constraints: A succinate production case study. Metab Eng 8:46–57.

Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645.

de Graef MR, Alexeeva S, Snoep JL, de Mattos MJ. 1999. The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolicadaptation in *Escherichia coli*. J Bacteriol 181:2351–2357.

Du C, Lin SK, Koutinas A, Wang R, Webb C. 2007. Succinic acid production from wheat using a biorefining strategy. Appl Microbiol Biotechnol 76(6):1263–1270.

Egyud LG, Szent-Gyorgyi A. 1966. On the regulation of cell division. Proc Natl Acad Sci USA 56:203–207.

Glassner DA, Datta R. 1992. Process for the production and purification of succinic acid. Patent US 5,143,834.

Gokarn RR, Eiteman MA, Altman E. 2000. Metabolic analysis of *Escherichia coli* in the presence and absence of carboxylating enzymes phosphoenolpyruvate carboxylase and pyruvate carboxylase. Appl Environ Microbiol 66:844–1850.

Grabar TB, Zhou S, Shanmugam KT, Yomano LP, Ingram LO. 2006. Methylglyoxal bypass identified as source of chiral contamination in L(+) and D(−) lactate fermentations by recombinant *Escherichia coli*. Biotechnol Lett 28:1527–1535.

Guettler MV, Jain MK, Rumler D. 1996a. Method for making succinic acid, bacterial variants for use in the process, and methods of obtaining variants. Patent US 5,573,931.

Guettler MV, Jain MK, Soni BK. 1996b. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. Patent US 5,505,004.

Guettler MV, Jain MK, Soni BK. 1998. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. Patent US 5,723,322.

Hopper DJ, Cooper RA. 1971. The regulation of *Escherichia coli* methylglyoxal synthase: A new control site in glycolysis. FEBS Lett 13: 213–216.

Iverson TM, Luna-Chavez C, Croal LR, Cecchini G, Rees DC. 2002. Crystallographic studies of the *Escherichia coli* quinol-fumarate reductase with inhibitors bound to the quinol-binding site. J Biol Chem 277:16124–16130.

Keseler IM, Collado-Vides J, Gamma-Castro S, Ingraham J, Paley S, Paulsen IT, Peralta-Gil M, Karp PD. 2005. Ecocyc: A comprehensive database resource for *Escherichia coli*. Nucleic Acids Res 33:D334–D337.

Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG. 2001. A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics 73:56–65.

Lee SY, Hong SH, Lee SH, Park SJ. 2004. Fermentative production of chemicals that can be used for polymer synthesis. Macromol Biosci 4:157–164.

Lee SJ, Lee DY, Kim TY, Kim BH, Lee J, Lee SY. 2005. Metabolic engineering of *Escherichia coli* for enhanced production of succinic acid, based on genome comparison and *in silico* gene knockout simulation. Appl Environ Microbiol 71:7880–7887.

Lee SJ, Song H, Lee SY. 2006. Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succic acid production. Appl Environ Microbiol 72(3):1939–1948.

Lin H, Bennett GN, San KY. 2005a. Chemostat culture characterization of *Esherichia coli* mutant strains metabolically engineered for aerobic succinate production: A study of the modified metabolic network based on metabolite profile, enzyme activity, and gene expression profile. Metab Eng 7:337–352.

Lin H, Bennett GN, San KY. 2005b. 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.

Lin H, Bennett GN, San KY. 2005c. Effect of carbon sources differing in oxidation state and transport route on succinate production in metabolically engineered *Escherichia coli*. J Ind Microbiol Biotechnol 32: 87–93.

Lin H, Bennett GN, San KY. 2005d. Fed-batch culture of a metabolically engineered *Escherichia coli* strain designed for high-level succinate production and yield under aerobic conditions. Biotechnol Bioeng 90:775–779.

Martinez A, Grabar TB, Shanmugam KT, Yomano LP, York SW, Ingram LO. 2007. Low salt medium for lactate and ethanol production by recombinant *Escherichia coli* B. Biotechnol Lett 29:397–404.

McKinlay JB, Vieille C, Zeikus JG. 2007. Prospects for a bio-based succinate industry. Appl Microbiol Biotechnol 76:727–740.

Meynial-Salles I, Dorotyn S, Soucaille P. 2007. A new process for the continuous production of succinic acid from glucose at high yield, titer, and productivity. Biotechnol Bioeng 99(1):129–135.

Millard CS, Chao YP, Liao JC, Donnelly MI. 1996. Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in *Escherichia coli*. Appl Environ Microbiol 62:1808–1810.

Miller JH. 1992. A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Plainview, NY: Cold Spring Harbor Laboratory Press p 456.

Moniruzzaman M, Lai X, York SW, Ingram LO. 1997. Extracellular melibiose and fructose are intermediates in raffinose catabolism during fermentation to ethanol by engineered enteric bacteria. J Bacteriol 179:1880–1886.

Nghiem PP, Donnelly MI, Millard CS, Stols L. 1999. Method for the production of dicarboxylic acids. Patent US 5,869,301.

Okino S, Inui M, Yukawa H. 2005. Production of organic acids by *Corynebacterium glutanicum* under oxygen deprivation. Appl Microbiol Biotechnol 68:475–480.

Posfai G, Koob MD, Kirkpatrick HA, Blattner FR. 1997. Versatile insertion plasmids for targeted genome manipulations in bacteria: Isolation, deletion, and rescue of the pathogenicity island LEE of the *Escherichia coli* O157: H7 genome. J Bacteriol 179:4426–4428.

Purvis JE, Yomano LP, Ingram LO. 2005. Enhanced trehalose production improves growth of *Escherichia coli* under osmotic stress. Appl Environ Microbiol 71:3761–3769.

Sanchez AM, Bennett GN, San KY. 2005a. Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity. Metab Eng 7:229–239.

Sanchez AM, Bennett GN, San KY. 2005b. Efficient succinic acid production from glucose through overexpression of pyruvate carboxylase in an *Escherichia coli* alcohol dehydrogenase and lactate dehydrogenase mutant. Biotechnol Prog 21:358–365.

Sanchez AM, Bennett GN, San KY. 2006. Batch culture characterization and metabolic flux analysis of succinate-producing *Escherichia coli* strains. Metab Eng 8:209–226.

Sanwal BD. 1970. Regulatory mechanisms involving nicotinamide adenine nucleotide as allosteric effectors: A control of glucose 6-phosphate dehydrogenase. J Biol Chem 245(7):1625–1631.

Sawers G, Bock A. 1988. Anaerobic regulation of pyruvate formate-lyase from *Escherichia coli* K-12. J Bacteriol 170:5330–5336.

Song H, Lee JW, Choi S, You JK, Hong WH, Lee SY. 2007. Effects of dissolved CO_2 levels on the growth of *Mannheimia succiniciproducens* and succinic acid production. Biotechnol Bioeng 98(6):1296–1304.

Stols L, Donnelly MI. 1997. Production of succinic acid through overexpression of NAD dependent malic enzyme in an *Escherichia coli* mutant. Appl Environ Microbiol 63:2695–2701.

Thomason L, Court DL, Bubunenko M, Constantino N, Wilson H, Datta S, Oppenheim A. 2005. Current protocols in molecular biology. In: Ausubel FM, Brent R, Klingston RE, Moore DD, Deidman JD, Smith JA, Struhl K, editors. Recombineering: genetic engineering in bacteria using homologous recombination. New York: John Wiley & Sons. p 1.16.1–1.16.21.

Underwood SA, Buszko ML, Shanmugam KT, Ingram LO. 2004. Lack of protective osmolytes limits final cell density and volumetric productivity of ethanologenic *Escherichia coli* KO11 during xylose fermentation. Appl Environ Microbiol 70(5):2734–2740.

Vemuri GN, Eiteman MA, Altman E. 2002a. Effects of growth mode and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol 68:1715–1727.

Vemuri GN, Eiteman MA, Altman E. 2002b. Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. J Ind Microbiol Biotechnol 28:325–332.

Wendisch VF, Bott M, Eikmanns BJ. 2006. Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. Curr Opin Microbiol 9: 1–7.

Wood BE, Yomano LP, York SW, Ingram LO. 2005. Development of industrial medium-required elimination of the 2,3-butanediol fermentation pathway to maintain ethanol yield in an ethanologenic strain of *Klebsiella oxytoca*. Biotechnol Prog 21:1366–1372.

Yun NR, San KY, Bennett GN. 2005. Enhancement of lactate and succinate formation in *adhE* or *pta-ackA* mutants of NADH dehydrogenase-deficient *Escherichia coli*. J Appl Microbiol 99:1404–1412.

Zeikus JG, Jain MK, Elankovan P. 1999. Biotechnology of succinic acid production and markets for derived industrial products. Appl Microbiol Biotechnol 51:545–552.

Zhang X, Jantama K, Moore JC, Shanmugam KT, Ingram LO. 2007. Production of L-alanine by metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol 77(2):355–366.

Zhou S, Shanmugam KT, Ingram LO. 2003. Functional replacement of the *Escherichia coli* D-(−)-lactate dehydrogenase gene (*ldhA*) with the L-(+)-lactate dehydrogenase gene (*ldhL*) from *Pediococcus acidilactici*. Appl Environ Microbiol 69:2237–2244.

Zhou S, Grabar TB, Shanmugam KT, Ingram LO. 2006a. Betaine tripled the volumetric productivity of D-(−)-lactate by *Escherichia coli* strain SZ132 in mineral salts medium. Biotechnol Lett 28:671–676.

Zhou S, Shanmugam KT, Yomano LP, Grabar TB, Ingram LO. 2006b. Fermentation of 12% (w/v) glucose to 1.2 M lactate by *Escherichia coli* strain SZ194 using mineral salts medium. Biotechnol Lett 28: 663–670.