APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2006, p. 1939–1948 Vol. 72, No. 3
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.3.1939–1948.2006

Genome-Based Metabolic Engineering of *Mannheimia succiniciproducens* for Succinic Acid Production‡

Sang Jun Lee,[1,2]† Hyohak Song,[1]† and Sang Yup Lee[1,2,3]*

Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical and Biomolecular Engineering,[1] Center for Ultramicrochemical Process Systems,[2] and Department of BioSystems, BioProcess Engineering Research Center and Bioinformatics Research Center,[3] Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea

Received 7 November 2005/Accepted 3 January 2006

Succinic acid is a four-carbon dicarboxylic acid produced as one of the fermentation products of anaerobic metabolism. Based on the complete genome sequence of a capnophilic succinic acid-producing rumen bacterium, *Mannheimia succiniciproducens*, gene knockout studies were carried out to understand its anaerobic fermentative metabolism and consequently to develop a metabolically engineered strain capable of producing succinic acid without by-product formation. Among three different CO_2-fixing metabolic reactions catalyzed by phosphoenolpyruvate (PEP) carboxykinase, PEP carboxylase, and malic enzyme, PEP carboxykinase was the most important for the anaerobic growth of *M. succiniciproducens* and succinic acid production. Oxaloacetate formed by carboxylation of PEP was found to be converted to succinic acid by three sequential reactions catalyzed by malate dehydrogenase, fumarase, and fumarate reductase. Major metabolic pathways leading to by-product formation were successfully removed by disrupting the *ldhA*, *pflB*, *pta*, and *ackA* genes. This metabolically engineered LPK7 strain was able to produce 13.4 g/liter of succinic acid from 20 g/liter glucose with little or no formation of acetic, formic, and lactic acids, resulting in a succinic acid yield of 0.97 mol succinic acid per mol glucose. Fed-batch culture of *M. succiniciproducens* LPK7 with intermittent glucose feeding allowed the production of 52.4 g/liter of succinic acid, with a succinic acid yield of 1.16 mol succinic acid per mol glucose and a succinic acid productivity of 1.8 g/liter/h, which should be useful for industrial production of succinic acid.

Succinic acid, also known as butanedioic or amber acid, is a four-carbon dicarboxylic acid synthesized as an intermediate of the tricarboxylic acid cycle and also as one of the mixed-acid fermentation products. There is growing interest in the production of succinic acid from renewable resources by microbial fermentation because succinic acid can be used in numerous applications (36, 37). Some anaerobic bacteria, such as *Anaerobiospirillum succiniciproducens* (6) and *Actinobacillus succinogenes* (10), produce succinic acid as a major fermentation product of their metabolism. During anaerobic fermentation, the greenhouse gas CO_2 is fixed by carboxylation reactions, converting C_3 to C_4 metabolites. Although *A. succiniciproducens* and *A. succinogenes* are able to produce relatively more succinic acid than other microorganisms, concomitant production of metabolic by-products, such as acetic, formic, and lactic acids, is problematic because it reduces the succinic acid yield and makes the purification process difficult and costly.

With an aim to reduce by-product formation, several different approaches have been taken. In one example, Guettler et al. (9) developed a monofluoroacetate-resistant mutant strain of *A. succinogenes* that produced less acetic and formic acids than the parent strain. In other studies, Lee et al. used glycerol as a carbon source (22) and supplied hydrogen gas (21) during fermentation to reduce by-product formation and simultaneously enhance succinic acid production by *A. succiniciproducens*. However, production of mixed acids still persisted.

Succinic acid production by recombinant *Escherichia coli* has also been intensively studied in the last decade (5, 12–14, 31, 34). Successful metabolic engineering of *E. coli* resulted in substantial increases in the succinic acid yield and productivity (5, 12, 13, 24, 28, 31, 34, 35). Vemuri et al. (35) reported an impressive result, achieving 99 g/liter of succinate by fed-batch fermentation of recombinant *E. coli*. However, relatively large amounts of acetic acid and ethanol were concomitantly produced, which is a problem to be solved.

Recently, a gram-negative capnophilic bacterium, *Mannheimia succiniciproducens* MBEL55E, was isolated from the rumens of Korean cows (20). *M. succiniciproducens* was able to produce a large amount of succinic acid as a major fermentation product under anaerobic conditions in the presence of CO_2. More recently, we reported the complete genome sequence of *M. succiniciproducens* and in silico genome scale metabolic characteristics (11). The genome scale metabolic-flux analysis showed that phosphoenolpyruvate (PEP) carboxylation is a major CO_2-fixing step (Fig. 1), and this flux has a direct relationship with succinic acid flux in a branched tricarboxylic acid cycle (11). Having the complete genome sequence of *M. succiniciproducens* in hand, we examined the possibility of eliminating the by-product formation by metabolic engineering. Here, we report the strategies for the metabolic engineering of *M. succiniciproducens* based on the genome sequence and construction of an improved succinic acid producer with reduced by-product formation.

* Corresponding author. Mailing address: Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea. Phone: 82 42 869 3930. Fax: 82 42 869 3910. E-mail: leesy@kaist.ac.kr.

† These authors contributed equally to this work.

‡ Supplemental material for this article can be found at http://aem.asm.org/.

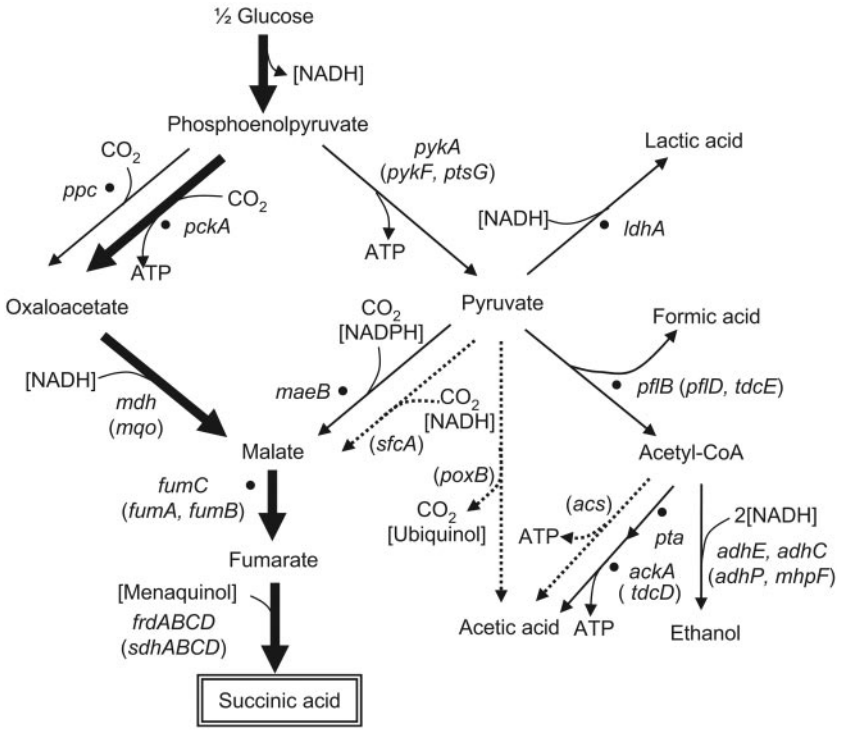

FIG. 1. Central fermentative metabolic pathways leading to the formation of mixed acids in *M. succiniciproducens* MBEL55E (solid arrows). The dashed arrows and parentheses indicate metabolic pathways and isoenzyme genes, respectively, which are not found in *M. succiniciproducens* but are found in *E. coli* K-12 (see Table S1 in the supplemental material). The filled circles indicate the genes disrupted for the characterization of metabolic-flux redistribution and the construction of a succinic acid overproducer. The boldface arrows indicate major metabolic pathways leading to succinic acid formation in *M. succiniciproducens*.

MATERIALS AND METHODS

Strains and media. All strains and plasmids used in this study are listed in Table 1. *E. coli* was cultivated in Luria-Bertani (LB) medium (10 g/liter Bacto tryptone, 5 g/liter yeast extract, and 10 g/liter sodium chloride) at 37°C. *M. succiniciproducens* MBEL55E (KCTC 0769BP; Korean Collection for Type Cultures, Daejeon, Korea) was routinely cultivated in LB medium supplemented with 10 g/liter of glucose at 37°C. Batch fermentations were carried out at 39°C (see below). When needed, chloramphenicol (Cm), kanamycin (Km), and spectinomycin (Sp) were added at concentrations of 34, 25, and 50 μg/ml, respectively, for *E. coli* and 6.8, 25, and 50 μg/ml, respectively, for *M. succiniciproducens*.

Plasmids. All restriction enzymes used in this work were purchased from New England Biolabs (Ipswich, MA). Ex *Taq* DNA polymerase (Takara, Shiga, Japan) was used for PCR. Genomic DNA was isolated from *M. succiniciproducens*

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
M. succiniciproducens MBEL55E	Bovine rumen isolate	20
M. succiniciproducens LK	*ldhA*::Km^r	This study
M. succiniciproducens LPK	*ldhA*::Km^r *pflB*::Cm^r	This study
M. succiniciproducens LPK2	*ldhA*::Km^r *pflB*::Cm^r *pckA*::Sp^r	This study
M. succiniciproducens LPK4	*ldhA*::Km^r *pflB*::Cm^r *ppc*::Sp^r	This study
M. succiniciproducens LPK5	*ldhA*::Km^r *pflB*::Cm^r *maeB*::Sp^r	This study
M. succiniciproducens LPK6	*ldhA*::Km^r *pflB*::Cm^r *fumC*::Sp^r	This study
M. succiniciproducens LPK7	*ldhA*::Km^r *pflB*::Cm^r *pta-ackA*::Sp^r	This study
E. coli XL1-Blue	Cloning host	Stratagene
pUC18	2.7 kb; Ap^r; cloning vector	New England Biolabs
pUC19	2.7 kb; Ap^r; cloning vector	New England Biolabs
pACYC184	4.2 kb; Cm^r Tet^r; chloramphenicol resistance gene	New England Biolabs
pUC4K	4.0 kb; Ap^r Km^r; kanamycin resistance gene	Pharmacia
pKmobsacB	5.7 kb; Km^r *sacB*; levansucrase gene	29
pIC156	4.0 kb; Sp^r; spectinomycin resistance gene	30
pLDHK-sacB	7.8 kb; Ap^r Km^r *sacB*; *ldhA* disruption vector	This study
pPFLC-sacB	8.0 kb; Ap^r Cm^r *sacB*; *pflB* disruption vector	This study
pPPCS-sacB	7.8 kb; Ap^r Sp^r *sacB*; *ppc* disruption vector	This study
pPCKS-sacB	7.8 kb; Ap^r Sp^r *sacB*; *pckA* disruption vector	This study
pMAES-sacB	8.0 kb; Ap^r Sp^r *sacB*; *maeB* disruption vector	This study
pFUMS-sacB	7.8 kb; Ap^r Sp^r *sacB*; *fumC* disruption vector	This study
pPTAS-sacB	8.0 kb; Ap^r Sp^r *sacB*; *pta* and *ackA* gene disruption vector	This study

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′→3′)[a]	Comments
P1	CAGTGAAGGAGCTCCGTAACGCATCCGCCG	SacI
P2	CTTTATCGAATCTGCAGGCGGTTTCCAAAA	PstI
P3	GTACTGTAAACTGCAGCTTTCATAGTTAGC	PstI
P4	GCCGAAAGTCAAGCTTGCCGTCGTTTAGTG	HindIII
P5	TCTAGAAGCT	XbaI
P6	GCTCTAGACCTTCTATCGCCTTCTTGACG	XbaI
P7	GCTCTAGAGGCTACAAAATCACGGGCGTC	XbaI
P8	AGCGGATCCCCTTCTATCGCCTTCTTGACG	BamHI
P9	GTCCTGCAGGGCTACAAAATCACGGGCGTC	PstI
P10	CATGGCGGATCCAGGTACGCTGATTTCGAT	BamHI
P11	CAAGGATCCAACGGATAAAGCTTTTATTAT	BamHI
P12	CTCGAGCCCGGGGTTTAAGGGCACCAATAA	SmaI
P13	CTCGAGCCCCGGGCTTTGCGCCGAATAAAT	SmaI
P14	TACGGATCCCCAGAAAATCGCCCCCATGCCGA	BamHI
P15	GCTCTAGATATCGTTTGATATTGTTCCGCCACATTTG	XbaI, EcoRV
P16	CGGGATCCGATATAAAGCGATGTTCTTGCTTGCCCAAT	BamHI
P17	GCTCTAGATATCATTATTGGAAAATATATGAAAATAA	XbaI, EcoRV
P18	CGGGATCCACCTGCAGATCACGGGCAATATTC	BamHI
P19	GCTCTAGATATCATAATTTTAGTAGCGAATTCCGGCTGCG	XbaI, EcoRV
P20	GCTCTAGATATCGACAAATGGGTGGTACCTGCCGATATGG	XbaI, EcoRV
P21	GTGGTGGATCCTAAACGTTCCGAAGCCTTGTTATCC	BamHI
P22	GCTCTAGATATCCGCAGTATCACTTTCTGCGC	XbaI, EcoRV
P23	TCCGCAGTCGGATCCGGGTTAACCGCACAG	BamHI
P24	GCTCTAGATATCCGTCAGGAAAGCACCCGCCATAGC	XbaI, EcoRV
P25	GGGGAGCTCGTGTGGCGCTGCGGAAGTAAGGCACAAAATC	SacI
P26	GCTCTAGATATCTATGGCGCTATCAAACGTGA	XbaI, EcoRV
P27	GGGGAGCTCATTTGTTATACAGATTAAATAATTTTTGCC	SacI
P28	GCTCTAGATATCCGACTACAATCAACGAAGAAATGAAAATG	XbaI, EcoRV
P29	GGGGAGCTCACGCCACTTGCGGTTCAATACCGAAACGG	SacI
P30	CCAGTCCATACCGATTTCCTGTGCGAGCTC	SacI
P31	GCTCTAGATATCTGCAGGAACCTGCACTTCACCCATAG	XbaI, EcoRV
P32	GGGGAGCTCGCTAACTTAGCTTCTAAAGGCCATGTTTCC	SacI
P33	GCTCTAGATATCCGGGTCAATATCGCCGCAAC	XbaI, EcoRV
P34	GCTCGCCCGGGGATCGATCCTCTAGAGT	SmaI
P35	CCCCCCCGGGCCGACAGGCTTTGAAGCATG	SmaI
P36	GACGTTTCCCGTTGAATATGGC	*KmR* 5′ end
P37	CATTGAGGCGTATTATCAGGAAAC	*ldhA* upstream
P38	GCAGTTTCATTTGATGCTCGATG	*KmR* 3′ end
P39	CCTCTTACGATGACGCATCTTTCC	*ldhA* downstream
P40	GGTGGTATATCCAGTGATTTTTTTCTCCAT	*CmR* 5′ end
P41	CTTTGCAACATTATGGTATGTATTGCCG	*pflB* upstream
P42	TACTGCGATGAGTGGCAGGGCGGGGCGTAA	*CmR* 3′ end
P43	CCCCAGCATGTGCAAATCTTCGTCAC	*pflB* downstream
P44	TCAAATTGCTCATGATTTCACCTCGTTG	*SpR* 5′ end
P45	GATCCAGGGAATGGCACGCAGGCTTTCAACGCCGCC	*ppc* upstream
P46	GGCTATCGGGGAGAGTGTGATGATAAGTGG	*SpR* 3′ end
P47	GCAAAGCCAGAGGAATGGATGCCATTAACCAATAGCG	*ppc* downstream
P48	GGCGTATTTCAGGCAGACGTAGGCGATGCAACCGAC	*pckA* upstream
P49	GCTTTACCGCCATCTTGGACTAAGGTTGCGGCAAG	*pckA* downstream
P50	GAGCAGATTTGCCGCGCCTTTGAGCGGACTGTCAGGC	*maeB* upstream
P51	GGTTTGCGCCCGTAGCTATCGCCCACCGGTCCCCAG	*maeB* downstream
P52	CGATACCCTGTGCCGCTCGTGCCGAAAGCTTGGCGG	*fumC* upstream
P53	GCGGTGTAGGTTTGGGCGGTTATTGGGCTGAGCGGG	*fumC* downstream
P54	GCTGCCAAACAACCGAAAATACCGCAATAAACGGC	*pta-ackA* upstream
P55	GCAACGCGAGGGTCAATACCGAAGGATTTCGCCG	*pta-ackA* downstream

[a] Restriction sites are underlined.

MBEL55E using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The oligonucleotides used in this study are listed in Table 2. An *ldhA* gene disruption vector containing two homologous regions was constructed as follows. PCR was performed with oligonucleotide primers P1 and P2 using the genomic DNA of *M. succiniciproducens* as a template. The PCR product was digested with SacI and PstI and ligated into the SacI-PstI-digested pUC18 to make pLDH1 (see Fig. S1 in the supplemental material). Similarly, the PCR product obtained with primers P3 and P4 was digested with PstI and HindIII and ligated into the PstI-HindIII-digested pLDH1 to make pLDH12. The PstI-digested Km resistance gene cassette from pUC4K was inserted into the PstI-digested pLDH12 to make pLDH1K2. Linker P5 was inserted into SacI-digested pLDH1K2 to make a new XbaI site in the plasmid. To amplify the *sacB* gene, PCR was carried out using pKmobsacB (29) as a template and the oligonucleotide primers P6 and P7. Finally, the PCR product was digested with XbaI and inserted into the XbaI site of pLDH1K2 to make pLDHK-sacB for knocking out the *ldhA* gene (see Fig. S1 in the supplemental material).

To make a *pflB* gene disruption plasmid, the *sacB* gene was amplified with primers P8 and P9, digested with PstI and BamHI, and ligated into the PstI-BamHI-digested pUC19 to make pUC19-sacB. PCR was performed with primers P10 and P11 using the genomic DNA of *M. succiniciproducens* as a template. The

PCR product was digested with BamHI and ligated into the BamHI-digested pUC19-sacB to make pPFL12-sacB. To amplify a Cm resistance gene, PCR was performed with primers P12 and P13 using pACYC184 as a template. The SmaI-digested Cm resistance gene cassette was ligated into the Bst1107I-digested pPFL12-sacB to make pPFLC-sacB (see Fig. S2 in the supplemental material). To make the *ppc*, *pckA*, *maeB*, *fumC*, and *pta-ackA* gene disruption vectors, PCRs were performed with the primer pairs P14-P15, P16-P17, P18-P19, P20-P21, and P22-P23, respectively, using the genomic DNA of *M. succiniciproducens* as a template. The PCR products were subsequently digested with XbaI and BamHI and cloned into the XbaI-BamHI sites of pUC19 to make pPPC1, pPCK1, pMAE1, pFUM1, and pPTA1, respectively. Then, PCR products obtained with primer pairs P24-P25, P26-P27, P28-P29, P30-P31, and P32-P33 were digested with XbaI and SacI and cloned into the XbaI-SacI sites of pPPC1, pPCK1, pMAE1, pFUM1, and pPTA1 to make pPPC12, pPCK12, pMAE12, pFUM12, and pPTA12, respectively. The Sp resistance gene cassette was amplified from pIC156 (30) using primers P34 and P35, digested with SmaI, and inserted into the EcoRV sites of pPPC12, pPCK12, pMAE12, pFUM12, and pPTA12 to make pPPC1S2, pPCK1S2, pMAE1S2, pFUM1S2, and pPTA1S2, respectively. The orientation of Sp resistance genes in these plasmids was confirmed by PCR. Finally, the SacI-BamHI-digested DNA fragments of pPPC1S2, pPCK1S2, pMAE1S2, pFUM1S2, and pPTA1S2 were cloned into the SacI-BamHI sites of pUC19-sacB to make pPPCS-sacB, pPCKS-sacB, pMAES-sacB, pFUMS-sacB, and pPTAS-sacB, respectively (see Fig. S2 in the supplemental material).

Gene knockouts. *M. succiniciproducens* MBEL55E was grown to an optical density at 600 nm (OD_{600}) of 0.25. Cells from 100 ml of broth were harvested and washed twice with cold 10% (vol/vol) glycerol solution and resuspended in 1 ml of 10% (vol/vol) glycerol solution. Competent cells (50 μl) were mixed with 10 μl of plasmid DNA (5 μg/μl) isolated from *E. coli* XL1-Blue. Electroporation was performed using a Gene Pulser (Bio-Rad, Hercules, CA; 1.8 kV, 200 Ω, and 25 μF) and a 0.1-cm electrode gap cuvette (Bio-Rad). After incubation in 1 ml of LB broth containing 10 g/liter glucose at 37°C for 1.5 h, the cells were spread on an LB agar plate containing 10 g/liter glucose and 25 mg/liter of Km, 6.8 mg/liter of Cm, or 50 mg/liter of Sp, depending on the suicide vector employed. After incubation at 37°C for 48 h, transformants were randomly selected and transferred twice onto an LB agar plate containing 100 g/liter of sucrose and antibiotics as indicated above. Finally, several candidates were grown in LB medium containing 10 g/liter glucose, and their genomic DNAs were isolated for the confirmation of gene inactivation as follows. Disruption of the *ldhA* gene by double crossover was confirmed by PCRs using primer pairs P36-P37 and P38-P39. Primers P37 and P39 are located outside the cloned sequence in the pLDHK-sacB vector, and primers P36 and P38 are located in the Km resistance gene. Inactivation of the *pflB* gene by allelic exchange was confirmed by PCRs using primer pairs P40-P41 and P42-P43. Primers P41 and P43 are located outside the cloned sequence in the pPFLC-sacB vector, and primers P40 and P42 are located in the Cm resistance gene. Similarly, inactivation of the *ppc*, *pckA*, *maeB*, *fumC*, and *pta-ackA* genes was confirmed by PCRs using primer pairs P44-P45 and P46-P47, P44-P48 and P46-P49, P44-P50 and P46-P51, P44-P52 and P46-P53, and P44-P54 and P46-P55, respectively (Table 1).

Fermentation. *M. succiniciproducens* cells were routinely cultivated anaerobically in sealed bottles containing 250 ml of MH3 medium plus 5 g/liter of glucose and 1 mg/ml of $Na_2S \cdot 9H_2O$ under a CO_2 atmosphere. MH3 medium contains (per liter) 2.5 g polypeptone, 2.5 g yeast extract, 3 g K_2HPO_4, 1 g NaCl, 1 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$, and 3 g $MgCO_3$. Batch cultures were carried out in a 6.6-liter Bioflo 3000 fermentor (New Brunswick Scientific Co., Edison, NJ) containing 2.25 liters of modified MH3 (MMH3) medium plus 20 g/liter glucose. MMH3 medium contains (per liter) 5 g yeast extract, 1.25 g NaCl, 0.4 g $CaCl_2 \cdot 2H_2O$, 0.4 g $MgCl_2 \cdot 6H_2O$, 5.02 g K_2HPO_4, and 2.87 g KH_2PO_4. Fermentations were carried out at the optimal temperature of 39°C as reported previously (20). The pH was adjusted to 6.5 using 28% (vol/vol) ammonia solution. Foaming was controlled by the addition of Antifoam 289 (Sigma, St. Louis, MO). CO_2 gas, scrubbed free of oxygen by passing it through an oxygen trap (Agilent, Waldbronn, Germany), was sparged throughout the fermentation at a flow rate of 0.25 vol/vol/min. Agitation speed was controlled at 200 rpm. Fed-batch culture was carried out under the same conditions as batch cultures, using MMH3 medium containing 63 g/liter of glucose. A concentrated solution containing 700 g/liter glucose and 20 g/liter $MgSO_4 \cdot 7H_2O$ was fed into the fermentor using a peristaltic pump (Cole-Parmer, Vernon Hills, IL) when the glucose concentration in the fermentor decreased to ca. 30 g/liter, in order to maintain the glucose concentration at 30 to 45 g/liter.

Analytical procedures. The concentrations of glucose and organic acids were determined by high-performance liquid chromatography (ProStar 210; Varian, Palo Alto, CA) equipped with UV/visible-light (ProStar 320; Varian, Palo Alto, CA) and refractive index (Shodex RI-71, Tokyo, Japan) detectors. A MetaCarb 87H column (300 by 7.8 mm; Varian) was eluted isocratically with 0.01 N H_2SO_4 at 60°C at a flow rate of 0.6 ml/min. Cell growth was monitored by measuring the OD_{600} using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The cell concentration, defined as grams dry cell weight per liter, was calculated from the predetermined standard curve relating the OD_{600} to dry weight (an OD_{600} of 1 = 0.451 g dry cell weight/liter).

RESULTS

Development of the gene knockout method. Since appropriate genetic tools and plasmid vectors for engineering *M. succiniciproducens* were not available, we first developed a gene knockout method. Using pUC19 as a backbone plasmid, suicide vectors having the *sacB* gene, which has been used as a counterselectable marker (26) in many gram-negative bacteria and several gram-positive *Corynebacterium* species, were constructed to completely remove the functional open reading frames of the genes of interest from the chromosome.

The strategy employed for inactivating the *ldhA* and *pflB* genes, as examples, is depicted in Fig. 2. After the transformation of the plasmid pLDHK-sacB, cells that integrated the plasmid into the chromosome by single crossover were selected in a medium containing Km and glucose. Two types of integration, upstream and downstream of the *ldhA* gene, are possible, as shown in Fig. 2. Among the cells with the first crossover, those that lost the *ldhA* gene by the second crossover were selected in a medium containing Km and sucrose. Wild-type *M. succiniciproducens* has an invertase gene and can utilize sucrose as a carbon source. However, recombinant cells with the *sacB* gene cannot survive on high-sucrose medium, due to the accumulation of a levan polymer synthesized from sucrose by a levansucrase (encoded by the *sacB* gene). Cells that failed to remove the gene replacement plasmid containing the *sacB* gene by double crossover lysed on the agar plate containing a high concentration of sucrose.

The candidates with the *ldhA* gene disrupted were confirmed by PCRs using two primer pairs, P36-P37 and P38-P39. For the further inactivation of the *pflB* gene, the LK strain with the *ldhA* gene disrupted was transformed with pPFLC-sacB. Cells harboring the plasmid in their chromosome by the first crossover were selected in a medium containing Cm and glucose. Cells with the second crossover were selected in a medium containing Cm and sucrose. The candidates with *pflB* inactivated were confirmed by PCRs using two oligonucleotide pairs, P40-P41 and P42-P43. As a result, the LPK strain with *ldhA-pflB* disrupted was constructed. From this LPK strain, LPK2, LPK4, LPK5, LPK6, and LPK7 strains were similarly constructed using plasmids pPCKS-sacB, pPPCS-sacB, pMAES-sacB, pFUMS-sacB, and pPTAS-sacB, respectively. The respective transformants were selected in a medium containing Sp and glucose for the first crossover, followed by selection for the second crossover in a medium containing Sp and sucrose. The final knockout candidates were confirmed by PCRs using the primer sets listed in Table 2 (see also Materials and Methods).

Effects of disrupting the *ldhA and pflB* genes. The genes responsible for the formation of acetic, formic, lactic, and succinic acids in *M. succiniciproducens* MBEL55E were se-

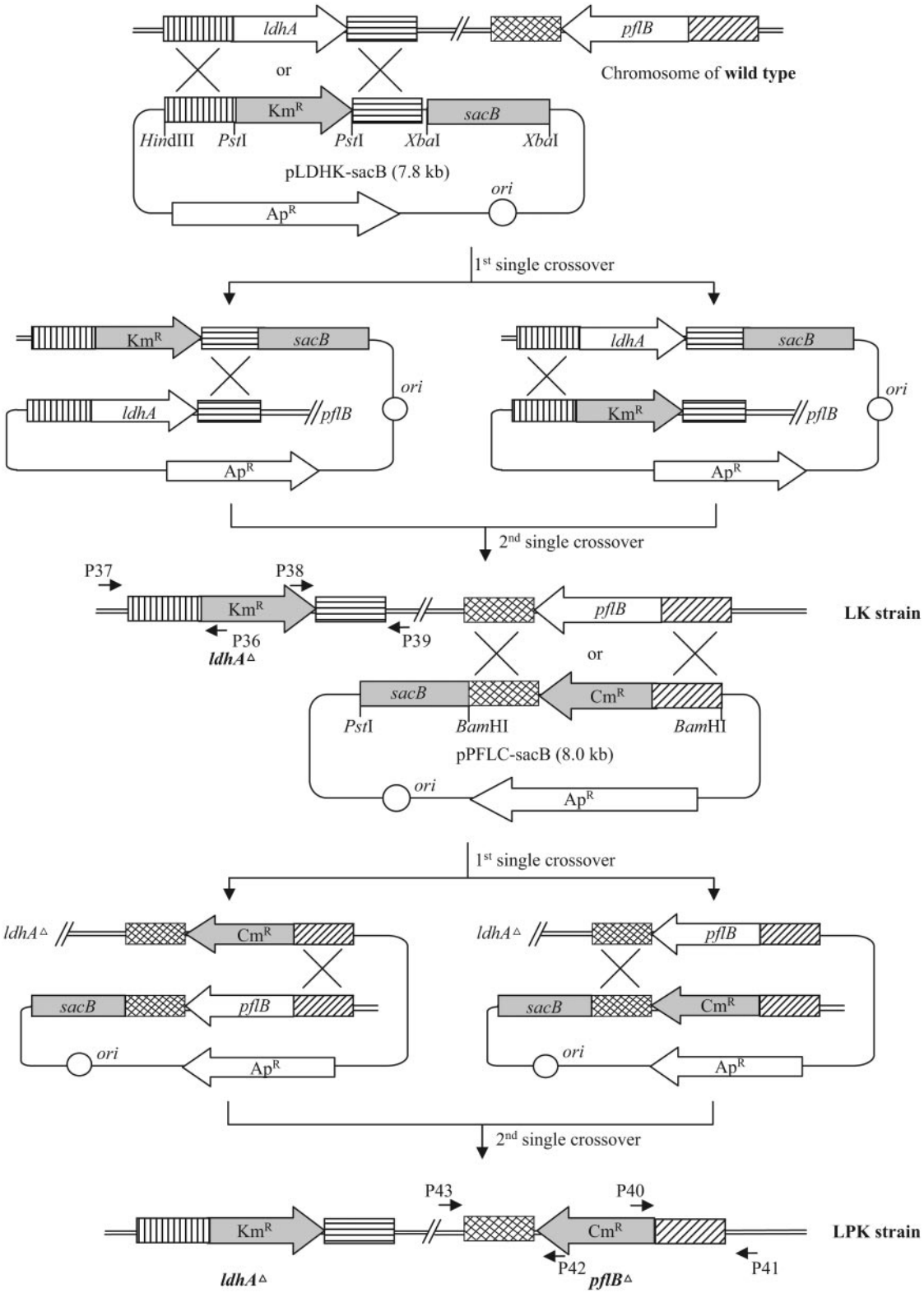

FIG. 2. Strategies for disrupting the *ldhA* and *pflB* genes in the chromosome of *M. succiniproducens* MBEL55E by using the integrative plasmids pLDHK-sacB and pPFLC-sacB, which contain the counterselectable levansucrase gene (*sacB*).

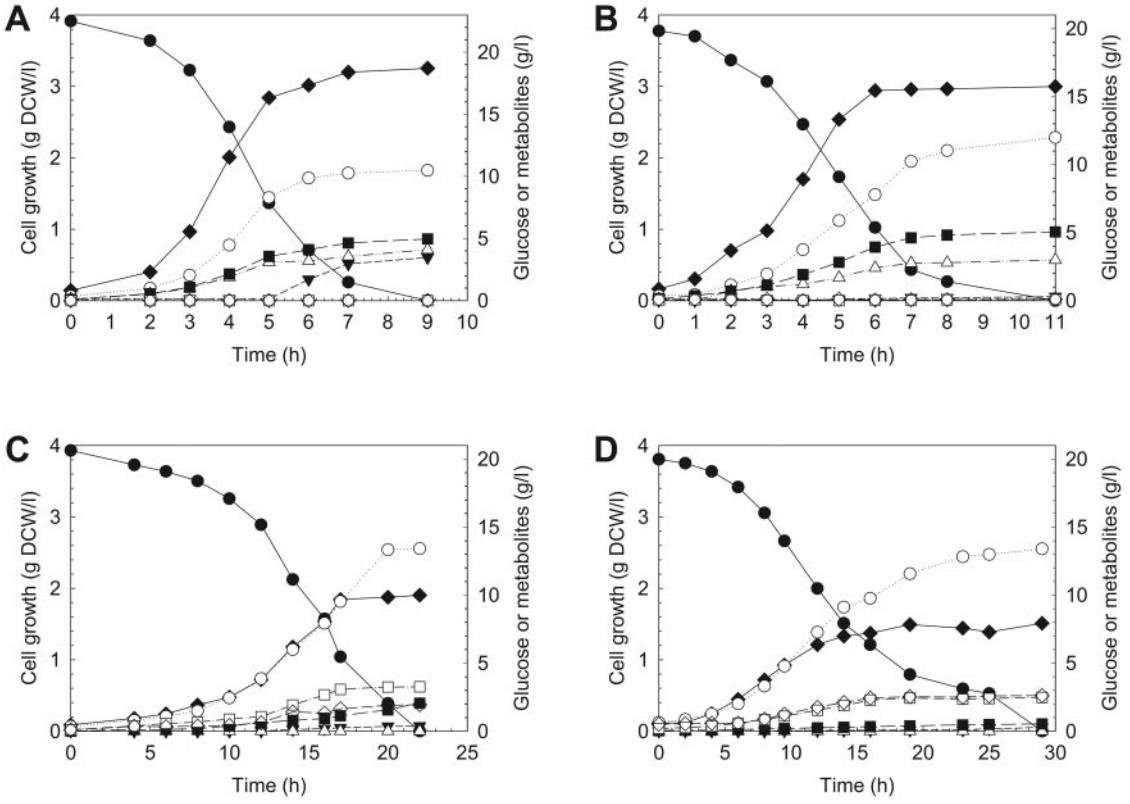

FIG. 3. Fermentation profiles of *M. succiniciproducens* MBEL55E (A), the LK strain with *ldhA* disrupted (B), the LPK strain with *ldhA-pflB* disrupted (C), and the LPK7 strain with *ldhA-pflB-pta-ackA* disrupted (D). ◆, cell growth; ●, glucose; ○, succinic acid; ▼, lactic acid; △, formic acid; ■, acetic acid; ◇, malic acid; □, pyruvic acid. DCW, dry cell weight.

lected from a functionally annotated genome database (15). The genes coding for the enzymes involved in the mixed-acid fermentative metabolism of *M. succiniciproducens* were compared with the known genes of *E. coli* K-12 (see Table S1 in the supplemental material). The key enzymes present in *E. coli*, but not in *M. succiniciproducens*, include a glucose-specific phosphotransferase system (PTS), NAD^+-dependent malic enzyme, membrane-associated malate dehydrogenase, fumarases A and B, pyruvate kinase I, pyruvate oxidase, and acetyl-coenzyme A synthetase (see Table S1 in the supplemental material). Our recent study suggested that *M. succiniciproducens* does not utilize the PTS for glucose uptake; rather, it possesses a glucokinase activity to transfer phosphate from ATP to glucose (23). The metabolic pathways leading to the formation of succinic acid and other organic acids in *M. succiniciproducens* under anaerobic condition are shown in Fig. 1.

Batch cultivation of wild-type *M. succiniciproducens* in MMH3 medium containing 22.5 g/liter glucose resulted in the production of 10.5 g/liter succinic acid, 4.96 g/liter acetic acid, 4.10 g/liter formic acid, and 3.47 g/liter lactic acid (Fig. 3A). In order to eliminate the formation of by-products, the genes responsible for the formation of acetic, formic, and lactic acids were sequentially knocked out. First, the *ldhA* gene was disrupted to prevent lactic acid formation. Anaerobic fermentation of the *ldhA*-deficient *M. succiniciproducens* LK strain showed much less lactic acid formation. Concomitantly, the final succinic acid concentration and yield increased to 12.0 g/liter and 0.90 mol succinic acid per mol glucose (Fig. 3B). Next, the *pflB* gene was additionally inactivated with an aim to eliminate the formation of acetic and formic acids. The formation of formic acid was completely eliminated (Fig. 3C). Production of acetic acid was significantly reduced in the LPK strain but still persisted. This suggests that there are other metabolic pathways contributing to the formation of acetic acid. Due to the inactivation of the major pyruvate dissimilation pathways and limited reducing power in the LPK strain, 3.26 g/liter of pyruvic acid and 1.95 g/liter of malic acid were produced. Also, the specific growth rate of the LPK strain (0.33 h^{-1}) was lower than those of the wild-type strain (0.88 h^{-1}) and the LK strain (0.59 h^{-1}). Nonetheless, it is notable that succinic acid formation increased to a final concentration of 13.4 g/liter with no formic acid and much less lactic acid production as a result of the *ldhA* and *pflB* inactivation (Fig. 3C). The succinic acid yield was 0.97 mol succinic acid per mol glucose (0.63 g succinic acid per g glucose).

Comparison of three CO_2-fixing pathways. Since succinic acid is a four-carbon dicarboxylic acid, carboxylation of PEP or pyruvate is an important step in its production. Three enzymes, PEP carboxylase (*ppc*) (18), PEP carboxykinase (*pckA*) (7), and malic enzyme (*maeB*) (4, 17), were considered to be responsible for the carboxylation of PEP or pyruvate in *M. succiniciproducens*. To examine which enzyme plays the most important role in C_3-C_4 conversion, the *maeB*, *ppc*, and *pckA* genes of *M. succiniciproducens* were separately disrupted in the

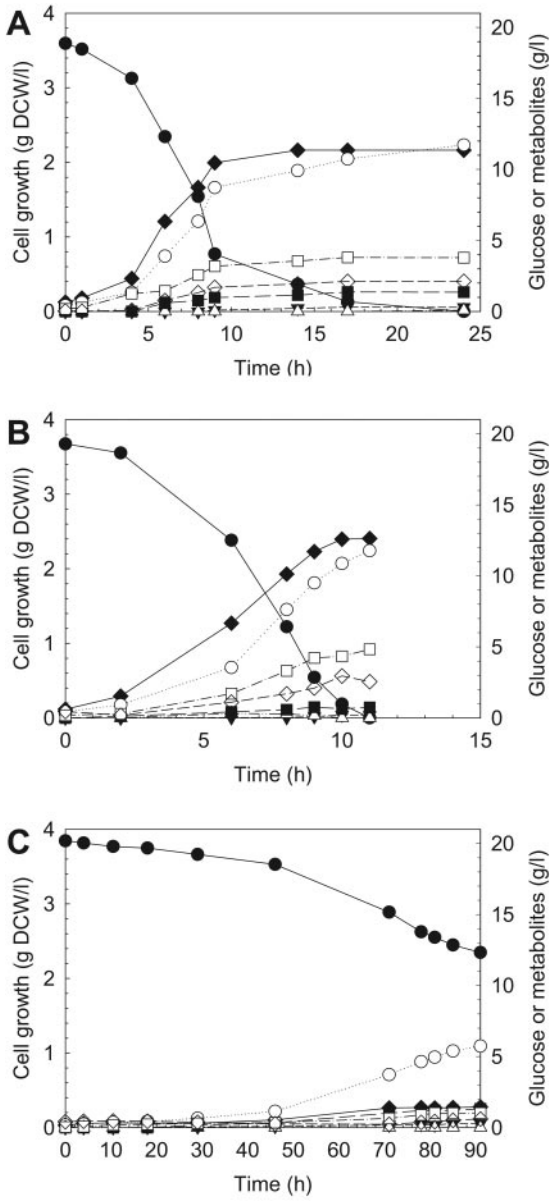

FIG. 4. Fermentation profiles of the *ldhA-pflB*-disrupted *M. succiniciproducens* mutant strains LPK5 (A), LPK4 (B), and LPK2 (C), which additionally lack the CO_2-fixing *maeB*, *ppc*, and *pckA* genes, respectively. ◆, cell growth; ●, glucose; ○, succinic acid; ▼, lactic acid; △, formic acid; ■, acetic acid; ◇, malic acid; □, pyruvic acid. DCW, dry cell weight.

LPK strain. The LPK5 strain with *ldhA-pflB-maeB* inactivated showed fermentation patterns similar to that of its parent LPK strain (Fig. 3C and 4A). The LPK4 strain with *ldhA-pflB-ppc* inactivated also showed a fermentation profile (Fig. 4B) similar to those of the LPK and LPK5 strains, except that it consumed glucose much more rapidly and produced more pyruvate (3.79 g/liter) and less acetic acid (1.36 g/liter). On the other hand, the LPK2 strain with *ldhA-pflB-pckA* inactivated showed very slow growth during the entire period of anaerobic fermentation, resulting in a significant decrease in succinic acid productivity (Fig. 4C and Table 3). These observations suggest that PEP carboxykinase encoded by the *pckA* gene plays the most important role in both anaerobic growth of *M. succiniciproducens* and efficient succinic acid production. Furthermore, the LPK6 strain, which is the LPK strain with *fumC* inactivated, could not grow under anaerobic conditions (data not shown), which suggests that formation of succinic acid is essential for the growth of the *M. succiniciproducens* LPK strain under anaerobic conditions. To our knowledge, this is the first report to present direct experimental evidence that the carbon flux efficiently flows from PEP to succinic acid via oxaloacetate, malate, and fumarate by metabolic pathways comprised of PEP carboxykinase, malate dehydrogenase, fumarase, and fumarate reductase in anaerobic succinic acid fermentation (Fig. 1).

Reducing acetic acid production. Inactivation of the *ldhA* and *pflB* genes almost completely blocked the formation of lactic and formic acids, but not acetic acid. With an aim to eliminate acetic acid formation, presumably contributed by unknown metabolic pathways, two key enzymes, phosphotransacetylase and acetate kinase, were further inactivated in the LPK strain to make the LPK7 strain. Acetic acid production was almost completely blocked in the LPK7 strain (Fig. 3D). The mass ratio of succinic acid to acetic acid and the succinic acid yield on glucose at the end of fermentation were 11.98- and 1.42-fold, respectively, higher than those obtained with a wild-type strain (Table 4). However, cell growth was somewhat negatively affected, presumably due to the shortage of ATP, which otherwise could be supplied by an acetate kinase reaction (Fig. 1). As observed with the LPK strain, pyruvic and malic acids were accumulated during the fermentation of the LPK7 strain, reaching 2.47 and 2.62 g/liter, respectively, at the end of fermentation (Table 4). They were not further converted to succinic acid even after prolonged fermentation (data not shown).

Fed-batch fermentation. Fed-batch fermentation of the *M. succiniciproducens* LPK7 strain was performed to achieve

TABLE 3. Effects of disruption of major CO_2-fixing enzymes in succinic acid fermentation by *M. succiniciproducens*

Strain	Concn of fermentation products (g/liter [mM])						Specific growth rate (h^{-1})	Maximum volumetric succinic acid productivity (g/liter/h)
	Succinic acid	Acetic acid	Formic acid	Lactic acid	Malic acid	Pyruvic acid		
LPK	13.42 (113.63)	2.06 (34.30)	ND[a]	0.37 (4.05)	1.95 (14.54)	3.26 (36.97)	0.33	1.59
LPK2	5.74 (48.57)	1.32 (21.98)	ND	0.30 (3.33)	0.60 (4.48)	1.02 (11.58)	0.04	0.11
LPK4	11.77 (99.68)	0.74 (12.35)	ND	0.19 (2.09)	2.55 (19.02)	4.83 (54.87)	0.40	1.98
LPK5	11.72 (99.25)	1.36 (22.68)	0.09 (1.89)	0.29 (3.23)	2.12 (15.84)	3.79 (43.00)	0.38	1.48

[a] ND, not detected.

TABLE 4. Comparison of fermentation results obtained with metabolically engineered *M. succiniciproducens* strains and wild-type strain

Strain	Consumed glucose concn (g/liter)	Biomass (g DCW/liter)	Concn of fermentation products (g/liter [mM])						Succinic acid mass yield (molar yield) on glucose	S/A mass ratio[a] (molar ratio)	Carbon recovery low (high) limit[b]	Redox balance[c]
			Succinic acid	Acetic acid	Formic acid	Lactic acid	Malic acid	Pyruvic acid				
MBEL55E	22.50	3.25	10.49 (88.8)	4.96 (82.6)	4.10 (88.9)	3.47 (38.4)	ND[d]	ND	0.45 (0.69)	2.11 (1.07)	1.01 (1.13)	1.19
LK	19.82	2.99	11.99 (101.5)	5.05 (84.0)	2.99 (65.0)	0.29 (3.10)	ND	0.08 (0.91)	0.59 (0.90)	2.37 (1.21)	1.01 (1.16)	1.10
LPK	20.63	1.90	13.42 (113.6)	2.06 (34.3)	ND	0.37 (4.00)	1.95 (14.5)	3.26 (36.9)	0.63 (0.97)	6.51 (3.31)	0.94 (1.13)	0.96
LPK7	19.98	1.50	13.40 (113.4)	0.53 (8.82)	ND	0.27 (3.00)	2.62 (19.5)	2.47 (28.0)	0.64 (0.97)	25.28 (12.8)	0.85 (1.05)	0.94
LPK7 fed batch	68.83	0.69[e]	52.43 (444.0)	0.81 (13.5)	ND	0.25 (2.70)	12.29 (91.67)	11.71 (132.9)	0.76 (1.16)	64.72 (32.8)	0.90 (1.14)	0.78

[a] Succinic acid (g) produced per g acetic acid produced (mole succinic acid produced per mole acetic acid produced).
[b] Carbon compound incorporated into the cell was calculated based on the formula $CH_{1.8}O_{0.6}N_{0.2}$ (25). The calculation did not take into account the amounts of metabolic products contained in the inocula, because they were only marginal. The low limit was calculated by assuming that all CO_2 incorporated came from external sources, while the high limit was calculated by assuming that all CO_2 incorporated was generated inside the cell. For the calculation of the low limit, carbon compound in succinic or malic acid was calculated as 3 mol carbon per mol acid, considering the fixation of 1 mol of external CO_2 per mol of succinic or malic acid. For the high limit, 4 mol carbon per mol succinic or malic acid was used, as no external CO_2 was considered. The actual carbon recovery value should be within these two limits.
[c] NADH (mole) produced per mole NAD^+ plus menaquinone produced. The redox balance was calculated based on the central carbon metabolism of *M. succiniciproducens* shown in Fig. 1.
[d] ND, not detected.
[e] The final cell concentration is rather low due to cell lysis toward the end of fed-batch culture (Fig. 5).

FIG. 5. Fed-batch fermentation profiles of the *M. succiniciproducens* LPK7 strain with *ldhA-pflB-pta-ackA* disrupted. ◆, cell growth; ●, glucose; ○, succinic acid; ▼, lactic acid; △, formic acid; ■, acetic acid; ◇, malic acid; □, pyruvic acid. DCW, dry cell weight.

higher succinic acid concentration and productivity (Fig. 5 and Table 4). A concentrated feeding solution containing glucose and $MgSO_4 \cdot 7H_2O$ was intermittently supplied when the glucose concentration in the fermentor decreased to ca. 30 g/liter. The constant volumetric succinic acid productivity of 2.97 g/liter/h, which is the highest succinic acid productivity reported so far, was observed for 10 to 25 h. Common by-products, including acetic, formic, and lactic acids, did not accumulate or accumulated only a little throughout the fermentation. Pyruvic and malic acids were the metabolic by-products produced instead. Cell growth stopped when the succinic acid concentration reached 36 g/liter at 19.5 h, after which the cells entered the death phase. However, succinic acid production continued, reaching 52 g/liter of succinic acid at 29 h, resulting in an overall succinic acid productivity of 1.8 g/liter/h (Fig. 5). The overall succinic acid yield was 1.16 mol succinic acid per mol glucose (0.76 g succinic acid per g glucose) (Table 4).

DISCUSSION

A capnophilic facultative anaerobic rumen bacterium, *M. succiniciproducens*, produces a significantly larger amount of succinic acid than other wild-type microorganisms during its anaerobic fermentation. In the genome of *M. succiniciproducens*, three CO_2-fixing enzymes encoded by the *pckA*, *ppc*, and *maeB* genes were identified in the central metabolic pathway. Even though metabolic pathways leading to the production of succinic acid in rumen bacteria have been discussed for *A. succinogenes* 130Z by comparing them with those of *E. coli* K-12 (33), direct evidence for the pathways leading to succinic acid formation has not been reported. We have demonstrated by systematic gene knockout studies that the *pckA-ldhA-pflB*-deficient strain showed severe growth defects (Fig. 4C). Furthermore, the *fumC-ldhA-pflB*-deficient strain could not grow anaerobically. Thus, it could be concluded that PEP is readily carboxylated by PEP carboxykinase to oxaloacetate, which is further converted to succinic acid by the sequential reactions catalyzed by malate dehydrogenase, fumarase, and fumarate reductase (Fig. 1).

Conversion of PEP to oxaloacetate by PEP carboxykinase provides two advantages in succinic acid production. First,

the PEP carboxylation reaction catalyzed by PEP carboxykinase produces ATP, which is not produced in the PEP carboxylase reaction. Kim et al. (19) reported that the overexpression of the *A. succinogenes pckA* gene resulted in enhanced succinic acid production in a *ppc*-negative *E. coli* strain. Second, the formation of other fermentation products is much less than for other wild-type microorganisms when PEP carboxylation is used. This is because pyruvate can be readily converted to acetic, formic, and lactic acids by pyruvate formate lyase and lactate dehydrogenase. Furthermore, the *M. succiniciproducens* LPK strain with *ldhA-pflB* disrupted showed much less of a growth defect than *E. coli* with *ldhA-pflB* disrupted, which showed almost no growth (32). This suggests that pyruvate dissimilation is not essential in the anaerobic cultivation of *M. succiniciproducens*, which is different from most microorganisms, including *E. coli*.

To further understand the carbon flux distributions, carbon recovery was calculated from the measured carbon content present in the form of organic acids in the culture broth (Table 4). Since the exact amounts of CO_2 fixation could not be determined, the carbon recovery values were given in ranges; the lower limit was calculated by assuming that all CO_2 used in the PEP carboxylation reaction was provided externally, while the higher limit was calculated by assuming that all CO_2 was generated inside the cell. Obviously, the actual carbon recovery value will be within this range. The highest fraction of carbon from glucose was converted to succinic acid in all fermentations, supporting the notion that *M. succiniciproducens* is one of the most promising succinic acid producers. The LPK7 strain with *ldhA*, *pflB*, and *pta-ackA* disrupted showed a dramatic increase in carbon recovery in the form of succinic acid (between the lower limit of 51% and the higher limit of 68%) at the expense of acetic, formic, and lactic acids. Furthermore, carbon recovery as succinic acid increased to 58% to 77% by fed-batch culture of LPK7.

The most notable development in this study was nearly complete elimination of common fermentation by-products, acetic, formic, and lactic acids. Since *M. succiniciproducens* does not possess the *poxB* and *acs* genes, the major acetic-acid-forming enzymes are phosphotransacetylase and acetate kinase, encoded by the *pta* and *ackA* genes, respectively (Fig. 1). However, a small amount of acetic acid (less than 0.53 g/liter) was still produced in the LPK7 strain, which lacks the *pta* and *ackA* genes. Bulter et al. (3) reported that *E. coli* still produced acetic acid even though all the enzymes encoded by the *pta*, *acs* and *poxB* genes responsible for acetic acid formation were simultaneously knocked out. This was attributed to known and unknown amino acid and fatty acid metabolism. Production of a small amount of acetic acid in *M. succiniciproducens* strain LPK7 seems to be due to the same reason. This could be supported by the finding that *M. succiniciproducens* possesses the *cysK* and *argE* genes, the products of which can form acetic acid in amino acid metabolism.

M. succiniciproducens possesses the *adhE* and *adhC* genes, which are known to be responsible for ethanol formation. They were highly similar to those of *E. coli* in amino acid sequence (see Table S1 in the supplemental material). When *M. succiniciproducens* was cultivated in a medium containing mannitol as the sole carbon source, a substantial amount of ethanol was produced (20), which certainly indicates the existence of ethanol-forming enzymes in the bacterium. However, ethanol formation was not observed when *M. succiniciproducens* was cultured on glucose, which is different from *E. coli*. Even though the reason for this phenomenon is not clear, the following explanation can be suggested. *M. succiniciproducens* does not use the PTS for glucose uptake (23) but uses a highly conserved mannitol-specific PTS for the uptake of mannitol. The mannitol-specific PTS gene is located upstream of the mannitol-1-phosphate dehydrogenase gene (*mtlD*) in the genome (see Table S1 in the supplemental material). When mannitol is used as a carbon source, pyruvate is formed during its transport. This might increase the intracellular concentration of pyruvate, which is subsequently converted to acetic acid and ethanol using the surplus reducing power generated by mannitol-1-phosphate dehydrogenase. Conversely, ethanol formation is not observed when glucose is used as a carbon source because of the limited pyruvate formation in *M. succiniciproducens*.

It was found that the metabolic intermediates pyruvic and malic acids were excreted when the major pyruvate-dissimilating enzymes lactate dehydrogenase and pyruvate formate lyase were simultaneously knocked out (as in strains LPK and LPK7). Pyruvate is formed from PEP by pyruvate kinase, encoded by the *pykA* gene, because the *pykF* and *ptsG* genes are not found in *M. succiniciproducens*. *M. succiniciproducens* seems to operate this reaction to produce the pyruvate and ATP required for cell growth. This was supported by the finding that the pyruvic acid concentration increased with cell growth and remained constant after cell growth stopped (Fig. 3C and D and 4A). Also, pyruvate can be formed from malate by malic enzyme (*maeB*). In *E. coli* and *Corynebacterium glutamicum*, the malic enzymes preferably convert malate to pyruvate, because the K_m values for malate and pyruvate are 0.4 and 16 mM and 3.8 and 13.8 mM, respectively (8, 12). Succinic acid is a highly reduced metabolite requiring four electrons per molecule. Excretion of pyruvic and malic acids in strains LPK and LPK7 suggests that cells are limited in reducing power for the formation of succinic acid.

Genome-based metabolic engineering allowed the development of an improved succinic acid producer with much reduced by-product formation. It is of course desirable to eliminate the production of pyruvic and malic acids to make a homofermentative succinic acid producer, which is our next goal. However, it should be mentioned that the separation of succinic acid from pyruvic and malic acids is much easier than with acetic, formic, and lactic acids. Huh et al. (16) reported that the amine-based extraction of succinic acid from fermentation broth is the most effective and economical method. A preliminary study using this method suggested that succinic acid can be purified much more efficiently when pyruvic and malic acids, rather than acetic, formic, and/or lactic acids, are present together (results not shown).

The succinic acid yield obtained by fed-batch culture was 1.16 mol succinic acid per mol glucose, which can be further increased by making more reducing power available and eliminating the formation of pyruvic and malic acids. Additional reducing power can be provided by directly supplying hydrogen gas (21), by using more reduced sugar as a carbon source (33), or by introducing or amplifying the enzymes that generate reducing power (1, 2). Also, coexpression of

the *sfcA* (or *maeB*) gene encoding malic enzyme (12, 31) and/or the *pyc* gene encoding pyruvate carboxylase (27, 34) can be considered for the efficient conversion of pyruvate to C_4 metabolite. The addition of a CO_2 source, such as $MgCO_3$, to the culture medium can further increase succinic acid formation. We did not add it in this study to avoid the formation of precipitates and thus to obtain more reproducible fermentation data, such as the OD_{600}.

In summary, the anaerobic fermentative metabolic characteristics of *M. succiniciproducens* were deciphered by genome-based metabolic engineering studies. Due to the lack of suitable genetic tools and plasmid vectors, the strategy of deleting the target genes from the chromosome was employed. An improved succinic acid producer could be developed by systematically disrupting the *ldhA*, *pflB*, *pta*, and *ackA* genes in the chromosome, which resulted in much reduced formation of common fermentation by-products.

ACKNOWLEDGMENTS

We thank Byung Hun Kim, Do Yun Kim, and Seong Jun Choi for their technical assistance. We also thank E. Dervyn (INRA, France) and J. K. Lee (KRIBB, Korea) for providing us with plasmids pIC156 and pKmobsacB, respectively.

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

REFERENCES

1. **Berrios-Rivera, S. J., G. N. Bennett, and K. Y. San.** 2002. Metabolic engineering of *Escherichia coli*: increase of NADH availability by overexpressing an NAD^+-dependent formate dehydrogenase. Metab. Eng. **4:**217–229.
2. **Berrios-Rivera, S. J., A. M. Sanchez, G. N. Bennett, and K. Y. San.** 2004. Effect of different levels of NADH availability on metabolite distribution in *Escherichia coli* fermentation in minimal and complex media. Appl. Microbiol. Biotechnol. **65:**426–432.
3. **Bulter, T., S. G. Lee, W. W. Wong, E. Fung, M. R. Connor, and J. C. Liao.** 2004. Design of artificial cell-cell communication using gene and metabolic networks. Proc. Natl. Acad. Sci. USA **101:**2299–2304.
4. **Chang, G. G., and L. Tong.** 2003. Structure and function of malic enzymes, a new class of oxidative decarboxylases. Biochemistry **42:**12721–12733.
5. **Chatterjee, R., C. S. Millard, K. Champion, D. P. Clark, and M. I. Donnelly.** 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.
6. **Davis, C. P., D. Cleven, J. Brown, and E. Balish.** 1976. *Anaerobiospirillum*, a new genus of spiral-shaped bacteria. Int. J. Syst. Bacteriol. **26:**498–504.
7. **Delbaere, L. T., A. M. Sudom, L. Prasad, Y. Leduc, and H. Goldie.** 2004. Structure/function studies of phosphoryl transfer by phosphoenolpyruvate carboxykinase. Biochim. Biophys. Acta **1697:**271–278.
8. **Gourdon, P., M. F. Baucher, N. D. Lindley, and A. Guyonvarch.** 2000. Cloning of the malic enzyme gene from *Corynebacterium glutamicum* and role of the enzyme in lactate metabolism. Appl. Environ. Microbiol. **66:** 2981–2987.
9. **Guettler, M. V., M. K. Jain, and D. Rumler.** November 1996. Method for making succinic acid, bacterial variants for use in the process, and methods of obtaining variants. U.S. patent 5,573,931.
10. **Guettler, M. V., M. K. Jain, and B. K. Soni.** April 1996. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. U.S. patent 5,504,004.
11. **Hong, S. H., J. S. Kim, S. Y. Lee, Y. H. In, S. S. Choi, J. K. Rih, C. H. Kim, H. Jeong, C. G. Hur, and J. J. Kim.** 2004. The genome sequence of the capnophilic rumen bacterium *Mannheimia succiniciproducens*. Nat. Biotechnol. **22:**1275–1281.
12. **Hong, S. H., and S. Y. Lee.** 2002. Importance of redox balance on the production of succinic acid by metabolically engineered *Escherichia coli*. Appl. Microbiol. Biotechnol. **58:**286–290.
13. **Hong, S. H., and S. Y. Lee.** 2001. Metabolic flux analysis for succinic acid production by recombinant *Escherichia coli* with amplified malic enzyme activity. Biotechnol. Bioeng. **74:**89–95.
14. **Hong, Y. K., W. H. Hong, and H. N. Chang.** 2000. Selective extraction of succinic acid from binary mixture of succinic acid and acetic acid. Biotechnol. Lett. **22:**871–874.
15. **Hou, B. K., J. S. Kim, J. H. Jun, D. Y. Lee, Y. W. Kim, S. Chae, M. Roh, Y. H. In, and S. Y. Lee.** 2004. BioSilico: an integrated metabolic database system. Bioinformatics **20:**3270–3272.
16. **Huh, Y. S., Y. K. Hong, W. H. Hong, and H. N. Chang.** 2004. Selective extraction of acetic acid from the fermentation broth produced by *Mannheimia succiniciproducens*. Biotechnol. Lett. **26:**1581–1584.
17. **Iwakura, M., J. Hattori, Y. Arita, M. Tokushige, and H. Katsuki.** 1979. Studies on regulatory functions of malic enzymes. VI. Purification and molecular properties of NADP-linked malic enzyme from *Escherichia coli* W. J. Biochem. **85:**1355–1365.
18. **Kai, Y., H. Matsumura, and K. Izui.** 2003. Phosphoenolpyruvate carboxylase: three-dimensional structure and molecular mechanisms. Arch. Biochem. Biophys. **414:**170–179.
19. **Kim, P., M. Laivenieks, C. Vieille, and J. G. Zeikus.** 2004. Effect of overexpression of *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase on succinate production in *Escherichia coli*. Appl. Environ. Microbiol. **70:** 1238–1241.
20. **Lee, P. C., S. Y. Lee, S. H. Hong, and H. N. Chang.** 2002. Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl. Microbiol. Biotechnol. **58:**663–668.
21. **Lee, P. C., W. G. Lee, S. Kwon, S. Y. Lee, and H. N. Chang.** 1999. Succinic acid production by *Anaerobiospirillum succiniciproducens*: effects of the H_2/CO_2 supply and glucose concentration. Enzyme Microb. Technol. **24:**549–554.
22. **Lee, P. C., W. G. Lee, S. Y. Lee, and H. N. Chang.** 2001. Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source. Biotechnol. Bioeng. **72:**41–48.
23. **Lee, S. J., D. Y. Lee, T. Y. Kim, B. H. Kim, J. Lee, and S. Y. Lee.** 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.
24. **Lin, H., G. N. Bennett, and K. Y. San.** 2005. 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.
25. **Mosey, F.** 1983. Mathematical modeling of the anaerobic digestion process: regulatory mechanisms for the formation of short-chain volatile acids from glucose. Water Sci. Technol. **15:**209–232.
26. **Reyrat, J.-M., V. Pelicic, B. Gicquel, and R. Rappuoli.** 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect. Immun. **66:**4011–4017.
27. **Sanchez, A. M., G. N. Bennett, and K. Y. San.** 2005. 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.
28. **Sanchez, A. M., G. N. Bennett, and K. Y. San.** 2005. Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity. Metab. Eng. **7:**229–239.
29. **Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
30. **Steinmetz, M., and R. Richter.** 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through *in vivo* recombination. Gene **142:**79–83.
31. **Stols, L., and M. Donnelly.** 1997. Production of succinic acid through overexpression of NAD^+-dependent malic enzyme in an *Escherichia coli* mutant. Appl. Environ. Microbiol. **63:**2695–2701.
32. **Stols, L., G. Kulkarni, B. G. Harris, and M. I. Donnelly.** 1997. Expression of *Ascaris suum* malic enzyme in a mutant *Escherichia coli* allows production of succinic acid from glucose. Appl. Biochem. Biotechnol. **63–65:**153–158.
33. **Van der Werf, M. J., M. V. Guettler, M. K. Jain, and J. G. Zeikus.** 1997. Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus* sp. 130Z. Arch. Microbiol. **167:**332–342.
34. **Vemuri, G. N., M. A. Eiteman, and E. Altman.** 2002. Effects of growth mode and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*. Appl. Environ. Microbiol. **68:**1715–1727.
35. **Vemuri, G. N., M. A. Eiteman, and E. Altman.** 2002. 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.
36. **Willke, T., and K. D. Vorlop.** 2004. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. Appl. Microbiol. Biotechnol. **66:**131–142.
37. **Zeikus, J. G., M. K. Jain, and P. Elankovan.** 1999. Biotechnology of succinic acid production and markets for derived industrial products. Appl. Microbiol. Biotechnol. **51:**545–552.