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Production of Optically Pure D-Lactic Acid in Mineral Salts Medium by Metabolically Engineered *Escherichia coli* W3110†

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The resistance of polylactide to biodegradation and the physical properties of this polymer can be controlled by adjusting the ratio of L-lactic acid to D-lactic acid. Although the largest demand is for the L enantiomer, substantial amounts of both enantiomers are required for bioplastics. We constructed derivatives of *Escherichia coli* W3110 (prototrophic) as new biocatalysts for the production of D-lactic acid. These strains (SZ40, SZ58, and SZ63) require only mineral salts as nutrients and lack all plasmids and antibiotic resistance genes used during construction. D-Lactic acid production by these new strains approached the theoretical maximum yield of two molecules per glucose molecule. The chemical purity of this D-lactic acid was ~98% with respect to soluble organic compounds. The optical purity exceeded 99%. Competing pathways were eliminated by chromosomal inactivation of genes encoding fumarate reductase (*frdABCD*), alcohol/aldehyde dehydrogenase (*adhE*), and pyruvate formate lyase (*pflB*). The cell yield and lactate productivity were increased by a further mutation in the acetate kinase gene (*ackA*). Similar improvements could be achieved by addition of 10 mM acetate or by an initial period of aeration. All three approaches reduced the time required to complete the fermentation of 5% glucose. The use of mineral salts medium, the lack of antibiotic resistance genes or plasmids, the high yield of D-lactate, and the high product purity should reduce costs associated with nutrients, purification, containment, biological oxygen demand, and waste treatment.

Lactic acid, a widely used specialty chemical in the food and pharmaceutical industries, has recently emerged as a potential bulk chemical for the production of renewable, biodegradable plastics from sugars (8, 17, 24). A Cargill Dow LLC manufacturing facility is currently under construction to produce up to 140,000 metric tons of polylactic acid per year, more than doubling the worldwide capacity for this chemical (26). Lactic acid has a chiral center and occurs as D-(−) and L-(+) enantiomers. Enantiomeric purity is important for industrial uses, and the greatest demand is for the L isomer. Deliberate blending of the enantiomers provides an effective method to control both the physical properties of polylactic acid (7, 44) and the rate of biodegradation (3, 19). The need for optically pure isomers favors the production of lactic acid monomers by biological processes rather than chemical processes which yield racemic mixtures (7, 44).

Further expansion of the lactic acid industry as a competitor of petroleum-based plastics will depend in part on the availability of low-cost sugar substrates, such as hexose and pentose sugars derived from lignocellulosic residues (4, 13, 24). Although many microorganisms produce lactic acid, commercially important strains, such as *Lactobacillus* strains, have been particularly useful due to their high acid tolerance and their ability to be genetically engineered for selective production of D-(−) or L-(+) optical isomers (5, 20, 28, 29). However, lactic acid bacteria also have undesirable traits, such as a requirement for complex nutrients which complicates acid recovery and incomplete or negligible pentose utilization (28, 29). Other promising biocatalysts are being developed for lactic acid production. These include strains of *Rhizopus* (41), *Bacillus* (33), *Escherichia* (11, 21), *Saccharomyces* (1), and *Kluyveromyces* (6, 36). Each biocatalyst, however, could benefit from one or more additional improvements, such as a broader substrate range, improved yield and productivity, reduction of nutritional requirements, elimination of plasmids and antibiotic markers, or improved optical purity of the product.

Escherichia coli, the workhorse of the biotechnology industry (13), can readily metabolize hexose and pentose sugars using only mineral salts as nutrients. During sugar fermentation, however, *E. coli* produces a mixture of organic acids (D-lactate, acetate, succinate, and formate) and ethanol to accommodate reducing equivalents generated during glycolysis (14, 18). Previous studies have demonstrated the feasibility of engineering *E. coli* for the production of L-lactate (11, 21) and D-lactate (11). The best *E. coli* strain reported for D-lactate production, strain JP203 (*pta*::Tn*5 phoA′-lacZ ppc*::*cat supE hsdS ara proA lacY galK rpsL xyl mtl*), contains multiple antibiotic resistance genes (*kan* and *tet*) and has an auxotrophic requirement for tricarboxylic acid pathway intermediates or amino acids and mutations blocking the utilization of pentoses and other sugars. Complex nutrients were used to evaluate D-lactate production by JP203, due in part to the complex nutritional requirements resulting from inactivation of phosphoenolpyruvate carboxylase (22, 43). During fermentation, high concentrations of D-lactate (63 g/liter; 700 mmol/liter) were produced from approximately 10% glucose with a volumetric productivity of 1 g/liter per h (11 mmol/liter per h). Although considerable glucose remained at the end of fermentation, the yields based on the amount of glucose metabolized ranged from 70 to 80% on a carbon basis (the maximum theoretical yield is 100%).

In this study, we developed new biocatalysts that convert

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
W3110	Wild type	ATCC 27325
TOP10F′	*lacI*^{q} (episome)	Invitrogen
DH5α	Δ*lacZ*M15 *recA*	Bethesda Research Laboratory
S17-1	*thi pro recA hsdR* RP4-2-*tet*::Mu *aphA*::Tn*Z* λ*pir*	40
BW25113	*lacI*^{q} *rrnB* Δ*lacZ hsdR* Δ*araBAD* Δ*rhaBAD*	16
AH218	Δ(*focA-pflB*)::*FRT-kan-FRT*	This study
SE1706	Δ*frdBC zid*::Tn*10*	34
TC20	Δ*adhE*::*FRT-tet-FRT*	This study
SZ31	W3110, Δ(*focA-pflB*)::*FRT-kan-FRT* P1(AH218) × W3110	This study
SZ32	W3110, Δ(*focA-pflB*)::*FRT*	This study
SZ34	W3110, Δ(*frdBC zid*)::Tn*10* P1(SE1706) × W3110	This study
SZ35	W3110, Δ(*focA-pflB*)::*FRT* Δ*frdBC zid*::Tn*10* P1(SE1706) × SZ32	This study
SZ37	W3110, Δ*frdBC*	This study
SZ40	W3110, Δ(*focA-pflB*)::*FRT* Δ*frdBC*	This study
SZ57	W3110, Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*adhE*::*FRT-tet-FRT* P1(TC20) × SZ40	This study
SZ58	W3110, Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*adhE*::*FRT*	This study
SZ61	W3110, *ackA*::*FRT-tet-FRT*	This study
SZ62	W3110, Δ(*focA-pflB*)::*FRT* Δ*frdBC* Δ*adhE*::*FRT ackA*::*FRT-tet-FRT* P1(SZ61) × SZ58	This study
SZ63	W3110, Δ*focA-pflB*::*FRT* Δ*frdBC* Δ*adhE*::*FRT ackA*::*FRT*	This study
Plasmids		
pCR2.1-TOPO	*bla kan*, TOPO TA cloning vector	Invitrogen
pFT-A	*bla flp* temperature-conditional replicon	37
pKD4	*bla FRT-kan-FRT*	16
pKD46	*bla* γ β *exo* (red recombinase), temperature-conditional replicon	16
pLOI2065	*bla FRT-tet-FRT*	This study
pLOI2224	*kan*, integration vector with conditional R6K replicon	31
pLOI2302	pUC19 containing *Asc*I linkers inserted into blunt *Nde*I and *Sap*I sites	46
pLOI2372	*bla ackA*	This study
pLOI2373	*bla ackA*::*FRT-tet-FRT*	This study
pLOI2375	*bla ackA*::*FRT-tet-FRT* conditional R6K replicon	This study
pLOI2803	*bla kan* Δ*adhE*::*FRT-tet-FRT*	This study

sugars to D-lactic acid (optical purity, >99.8%) at 97 to 99% of the theoretical yield by using only mineral salts as nutrients. All antibiotic resistance markers and plasmids used during construction were eliminated in the production strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α, TOP10F′, and S17-1 were used as hosts for plasmid construction. *E. coli* TC20 (Δ*adhE*::*tet*), AH218 (Δ*focA-pflB*::*kan*), and SE1706 (Δ*frdBC zid*::Tn*10*) were used as sources of mutations to construct homolactic strains. During plasmid and strain construction, cultures were grown in Luria-Bertani broth or agar (39). Antibiotics were included as appropriate at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 5 or 10 μg/ml; and ampicillin, 50 μg/ml. Selection on fusaric acid plates was used to remove the Tn*10*-encoded *tet* gene (34). Fusaric acid plates contained (per liter) 5 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 50 mg of chlortetracycline, 15 g of agar, 100 ml of NaH_2PO_4 (725 mM), 5 ml of $ZnCl_2$ (20 mM), and 6 ml of fusaric acid (11.2 mM)). Homolactic acid-producing strains were maintained on M9 medium (32) containing 2% glucose and 1.5% agar. Broth cultures were grown in M9 medium containing either 1% glucose (tube experiments and seed cultures) or 5% glucose (pH-controlled fermentors).

Genetic methods. Standard methods were used for plasmid construction, transduction with phage P1, PCR, and sequencing (32, 39). Chromosomal DNA from W3110 served as a template to amplify the *adhE* and *ackA* genes with primers (ORFmers) obtained from Sigma-Genosys (The Woodlands, Tex.). PCR products were initially cloned into pCR2.1-TOPO by using TOP10F′ as the host.

A removable tetracycline cassette (*FRT-tet-FRT*) was constructed (pLOI2065) and was analogous to the kanamycin cassette (*FRT-kan-FRT*) in pKD4 (16). In both cassettes, flanking *FRT* sites are oriented in the same direction to allow efficient in vivo excision by FLP recombinanase (37). Plasmid pLOI2065 contains two *Eco*RI sites and two *Sma*I sites for isolation of the *FRT-tet-FRT* cassette.

Knockout mutations and chromosomal deletions were constructed by using procedures developed by Posfai et al. (37), Datsenko and Wanner (16), and Martinez-Morales et al. (31). Resistance markers with flanking *FRT* sites were used to facilitate deletion. Chromosomal integrations and deletions were verified by using appropriate antibiotic markers, PCR analysis, and analysis of fermentation products. Plasmid constructs were verified by sequencing relevant regions.

Deletion of *pflB*. A Δ*focA-pflB*::*FRT* mutation was constructed by using the method of Datsenko and Wanner (16). Hybrid primers were designed which are complementary to *E. coli* chromosomal genes and to the antibiotic cassette (*FRT-kan-FRT*) in pKD4. The sense primer (**TTACTCCGTATTTGCATAAAA ACCATGCGAGTTACGGGCCTATAA**GTGTAGGCTGGAGCTGCTTC) consisted of an initial 45 bp (boldface type) corresponding to the region from position −130 to position −85 of *focA*, followed by 20 bp (underlined) corresponding to the primer 1 region of pKD4. The antisense primer (**ATAGATTGA GTGAAGGTACGAGTAATAACGTCCTGCTGCTGTTCT**CATATGAATATCC TCCTTAG) consisted of an initial 45 bp (boldface type) of the C-terminal end of *pflB*, followed by 20 bp (underlined) corresponding to the primer 2 region of pKD4. The *FRT-kan-FRT* cassette was amplified by PCR by using these primers and pKD4 as the template. After purification, amplified DNA was electroporated into *E. coli* BW25113(pKD46). The resulting kanamycin-resistant recombinant, AH218, contained *FRT-kan-FRT* in the deleted region of *pflB* (46 bp remaining). A phage P1 lysate prepared from AH218 (Δ*pflB*::*FRT-kan-FRT*) was used to transfer this mutation into W3110 to produce strain SZ31 (Δ*pflB*:: *FRT-kan-FRT*). After verification of this mutation by analysis of PCR products, fermentation products, and antibiotic markers, the *kan* gene was removed from the chromosome with FLP recombinase by using a temperature-conditional helper plasmid (pFT-A). After removal of the helper plasmid, the resulting kanomycin-sensitive strain (Δ*focA-pflB*::*FRT*) was designated SZ32.

Deletion of *adhE*. To construct an *adhE* mutant, the coding region (2.68 kbp) was amplified by PCR and cloned into pCR2.1-TOPO. The central region of *adhE* (1.06 kbp) was deleted by using *Hin*cII (two sites) and was replaced with a 1.7-kbp *Sma*I fragment from pLOI2065 containing the *FRT-tet-FRT* cassette to produce pLOI2803. This plasmid was linearized by digestion with *Pvu*I and *Sca*I and served as a template to amplify (with *adhE* primers) the 3.17-kbp region

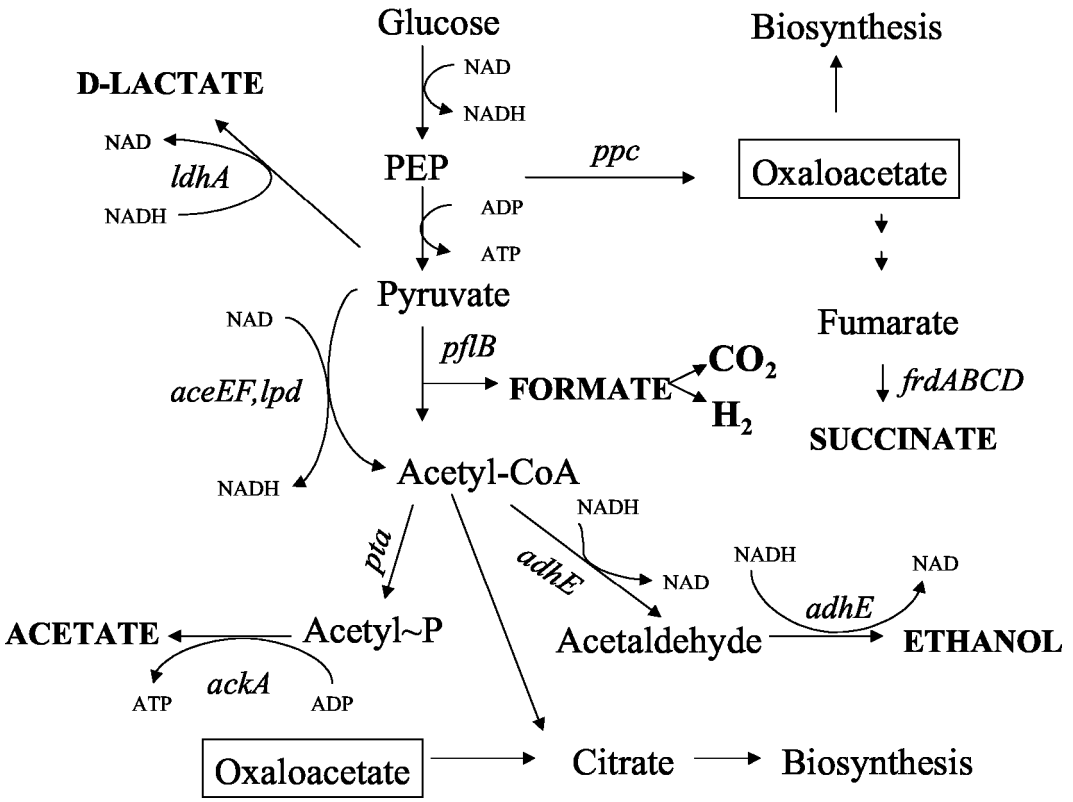

FIG. 1. Fermentation of glucose by *E. coli*. Primary fermentation products are indicated by boldface type. Genes encoding important enzymes are indicated by italics. For clarity, oxaloacetate (enclosed in a box) is shown twice, although it is presumed to exist as a single metabolic pool. PEP, phosphoenolpyruvate.

containing Δ*adhE*::*FRT-tet-FRT*. Amplified DNA was purified and introduced into W3110 by electroporation. Recombinants from double-crossover events were identified by using antibiotic markers and were confirmed by analysis of PCR and fermentation products. One clone was selected and designated TC20. Transduction with phage P1 was used to move this mutation from TC20 into SZ40, resulting in SZ57. The *tet* gene was deleted from SZ57 with FLP recombinase by using pFT-A (*flp*). After elimination of the helper plasmid by growth at 42°C, one resulting clone (Δ*focA-pflB*::*FRT* Δ*frdBC* Δ*adhE*::*FRT*) was designated SZ58.

Construction of *ackA* mutation. The *ackA* coding region was amplified, initially cloned into pCR2.1-TOPO, and subcloned (1.2-kbp *Eco*RI fragment) into the corresponding site of pLOI2302 to produce pLOI2372. A *Sma*I fragment from pLOI2065 containing *FRT-tet-FRT* (1.7 kbp) was inserted into the unique, dephosphorylated *Eco*RV site of pLOI2372 to produce pLOI2373. The 2.8-kbp *Asc*I fragment containing *ackA*::*FRT-tet-FRT* was isolated from pLOI2373 and cloned into the *Asc*I site of pLOI2224 (conditional R6K replicon) to complete the integration vector, pLOI2375. Plasmid pLOI2375 was introduced into W3110 by electroporation. Recombinants from double-crossover events were identified by antibiotic markers and were confirmed by analysis of PCR and fermentation products. One strain (*ackA*::*FRT-tet-FRT*) was selected and designated SZ61. The *ackA* mutation was then transferred from SZ61 into SZ58 by P1 transduction to produce SZ62 (Δ*focA-pflB*::*FRT* Δ*frd* Δ*adhE*::*FRT ackA*::*FRT-tet-FRT*). The *tet* gene was excised with FLP recombinase by using pFT-A. After removal of pFT-A by growth at 42°C, the resulting strain (Δ*focA-pflB*::*FRT* Δ*frd* Δ*adhE*::*FRT ackA*::*FRT*) was designated SZ63.

Fermentation. Cultures in 18-ml screw-cap tubes were used for the initial characterization of gene mutations concerned with fermentation. Single colonies from fresh plates were suspended in 1 ml of M9 medium and used to provide inocula (~50 μl) for tubes filled to the brim with M9 medium (1% glucose). Fermentation products were analyzed after incubation for 48 h at 37°C.

Seed cultures were prepared for larger (8-liter) fermentations by inoculating colonies from fresh M9 plates into 2-liter flasks containing 600 ml of M9 medium with 1% glucose. After incubation for 20 h (37°C, 200 rpm), a portion of each culture was harvested by centrifugation and used to inoculate a New Brunswick Bioflow 3000 fermentor (33.8 mg [dry weight] of cells per liter) containing 8 liters of M9 medium with 5% glucose. Mixing was provided by a single, midlevel, upflow marine impeller (37°C, 200 rpm). KOH (11.7 M) was automatically added to maintain the pH at 7.0. Samples were removed for analysis of organic acids, ethanol, and cell mass. Fermentations were terminated when base addition was no longer required to maintain the pH.

Analyses. Organic acid and residual glucose contents were determined by using a Hewlett-Packard high-performance liquid chromatograph (HPLC) (HP 1090 series II) equipped with UV (210-nm) and refractive index detectors. Products were separated by using a Bio-Rad HPX 87H column (injection volume, 10 μl) with 4 mM H_2SO_4 as the mobile phase (0.5 ml/min, 45°C). The identities of organic acids in fermentation broth were confirmed by nuclear magnetic resonance (45). The ethanol content was measured by gas chromatography (30). Optical isomers of D-(−)- and L-(+)-lactic acids were analyzed by using a Chiralpak MA+ column (Chiral Technologies, Exton, Pa.) as described by Omole et al. (35). Cell mass was estimated by measuring the optical density at 550 nm (1 liter of cells at an optical density at 550 nm of 3 was approximately equal to 1 g [dry weight] of cells) with a Bausch & Lomb Spectronic 70 spectrophotometer with round culture tubes (10 by 75 mm) as cuvettes.

Nucleotide sequence accession number. The nucleotide sequence of pLOI2065 has been deposited in the GenBank database under accession no. AF521666.

RESULTS

Construction of a homolactate fermentation pathway in *E. coli* W3110. Fermentation of sugars through native pathways in *E. coli* produces a mixture of organic acids, ethanol, CO_2, and H_2 (Fig. 1 and 2A). The distribution of carbon among these products is largely determined by the relative in vivo activities of D-lactate dehydrogenase (*ldhA* gene), pyruvate formatelyase (*pfl* gene), and phosphoenolpyruvate carboxylase

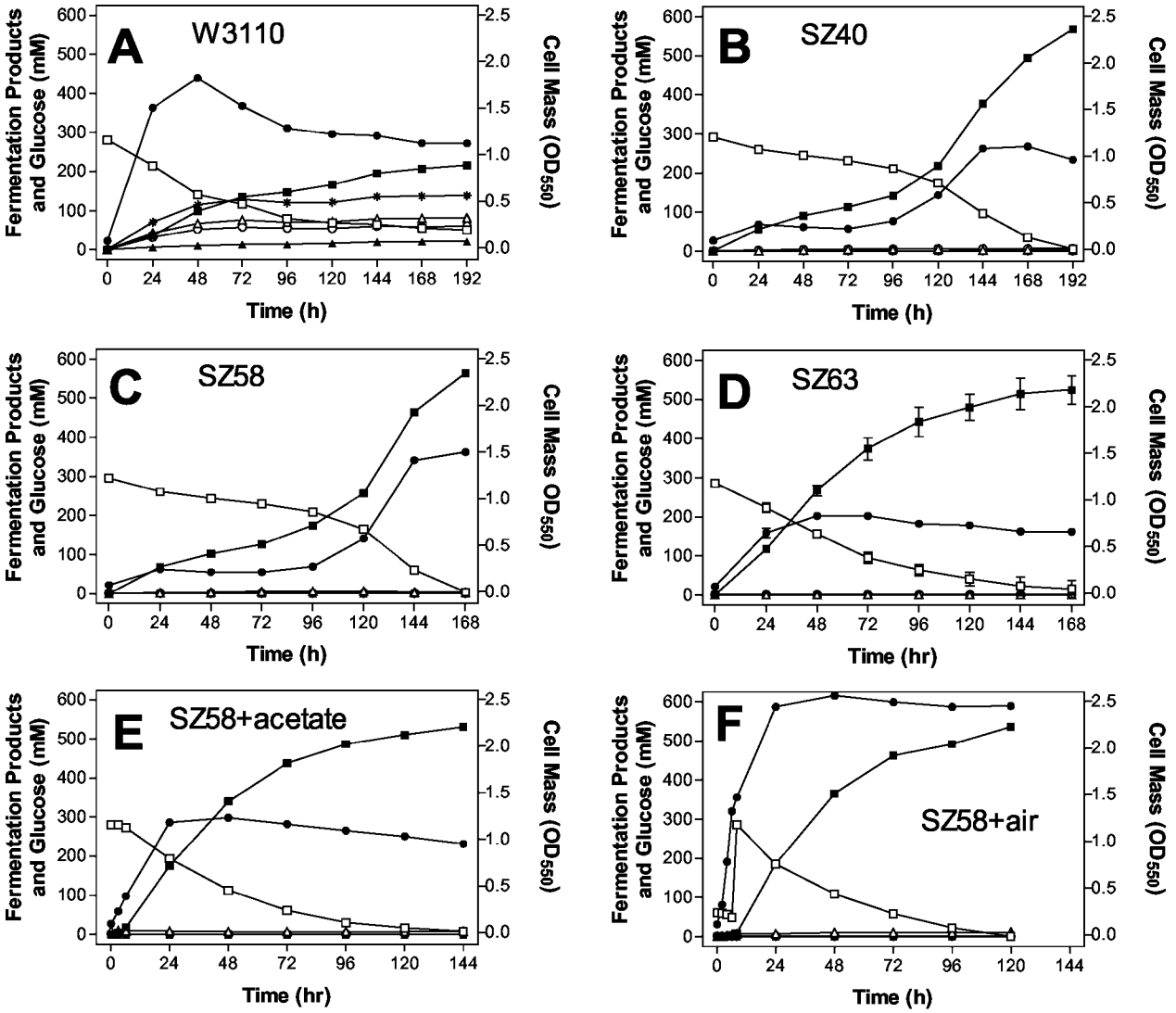

FIG. 2. Fermentation of 5% glucose by W3110 and derivatives of this strain. (A) W3110 (wild type); (B) SZ40; (C) SZ58; (D) SZ63; (E) SZ58 supplemented with 10 mM acetate; (F) SZ58 with 8 h of (initial) aeration. Symbols: ●, cell mass; □, glucose concentration; ■, lactate concentration; ▲, succinate concentration; ○, ethanol concentration; △, acetate; ∗, formate concentration. OD_{550}, optical density at 550 nm.

(*ppc* gene). Acetate and ethanol are typically produced in approximately equimolar amounts from acetyl coenzyme A (acetyl-CoA) to provide redox balance (14, 18). To construct a strain for D-lactate production, we used removable antibiotic resistance genes to sequentially inactivate chromosomal genes encoding alternative pathways.

A deletion was constructed in the *pflB* gene of W3110 to produce SZ32 (Δ*focA-pflB*::*FRT*). This single mutation eliminated the production of formate, ethanol, and acetate in tube cultures containing 1% glucose (Table 2). Several different mutations can be used to block succinate production (Fig. 1). During fermentation, the tricarboxylic acid pathway serves pri-

TABLE 2. Fermentation products from glucose[a]

Strain	Relevant mutations	Concn of fermentation products (mM)				
		Succinate	Lactate	Formate	Acetate	Ethanol
W3110	Wild type	9.36 ± 1.00[b]	30.69 ± 2.49	15.26 ± 0.34	18.42 ± 0.32	14.84 ± 1.77
SZ32	Δ*pflB*::*FRT*	13.23 ± 1.30	49.23 ± 1.26	<0.5	<0.5	<1.0
SZ37	Δ*frdBC*	<0.5	37.18 ± 9.05	12.95 ± 3.38	14.80 ± 1.33	13.42 ± 2.08
SZ40	Δ*pflB*::*FRT* Δ*frdBC*	<0.5	59.07 ± 0.69	<0.5	<0.5	<1.0

[a] Fermentation tests were conducted in 18-ml screw-cap culture tubes containing M9 medium supplemented with 1% glucose. Cultures were incubated for 48 h at 37°C.

[b] Mean ± standard deviation.

TABLE 3. Summary of fermentation results[a]

Strain (mutations and conditions)	Cell mass (mg/liter)	Amt of glucose utilized (mM)	Lactate concn (mM)	Yield (% of total; % utilized)[b]	Maximum volumetric productivity[c]		Maximum specific productivity[d]		Concn of coproducts (mM)			
					Rate (mmol/liter per h)	Time (h)	Rate (mmol/g [dry wt] of cells per h)	Time (h)	Succinate	Formate	Acetate	Ethanol
W3110	601	230	216	39; 47	2.33	48	4.46	24	21.5	138.2	81.2	60.5
SZ40 (*pflB frd*)	363	286	567	97; 99	6.41	192	27.71	120	<0.5	<0.5	4.4	7.0
SZ58 (*pflB frd adhE*)	495	292	564	95; 97	7.31	144	35.59	120	<0.5	<0.5	3.0	<1.0
SZ58 (*pflB frd adhE*; aeration)	785 ± 79	273 ± 11	505 ± 31	92; 93 ± 2	10.80 ± 1.6	24	15.58 ± 4.78	24	<0.5	<0.5	11.5 ± 0.6	<1.0
SZ58 (*pflB frd adhE*; 10 mM sodium acetate)	406	273	531	95; 97	9.11	72	44.63	48	<0.5	<0.5	7.13	<1.0
SZ63 (*pflB frd adhE ackA*)	284 ± 7	276 ± 15	539 ± 39	96; 98 ± 2	5.98 ± 0.70	24	27.28 ± 5.54	24	<0.5	<0.5	1.7 ± 0.3	<1.0

[a] The values are averages of two or more fermentations. Standard deviations were calculated when three or more replicates were used.
[b] Percentage of the maximum theoretical yield for lactic acid (2 mol of lactate per mol of glucose). The values are percentages of the total sugar added to the fermentation and percentages of the sugar which had been metabolized at the end of fermentation. Only the W3110 culture contained a significant amount of unmetabolized sugar after 192 h (50 mM).
[c] Maximum volumetric productivity for lactate.
[d] Maximum specific productivity for lactate.

marily as a source of carbon skeletons for biosynthesis. Inactivation of the *ppc* gene was previously utilized for construction of D-lactate-producing biocatalysts (11). However, inactivation of this gene has been shown to create an auxotrophic requirement for amino acids or dicarboxylic acids during growth in mineral salts medium with glucose (22, 43). Previous experience with *E. coli* B strains (25, 30) engineered for ethanol production showed that a deletion in the *frdABCD* operon (34, 42) can be used as an alternative method to block succinate production. P1 transduction was used to transfer this mutation (*frdBC zid*::Tn*10*) from SE1706 into SZ32, and the resulting strain was designated SZ35 (Δ*focA-pflB*::*FRT* Δ*frdBC zid*::Tn*10*). The *tet* gene was removed from SZ35 by fusaric acid selection to produce SZ40 (Δ*focA-pflB*::*FRT* Δ*frdBC*). This strain remained prototrophic and lacked all antibiotic resistance genes used during construction. For comparison, a W3110 derivative containing the Δ*frdBC* mutation alone (SZ37) was also constructed. Homolactate production by SZ40 was confirmed in tube cultures containing M9 medium with 1% glucose (Table 2); only lactate was produced as a fermentation product.

D-Lactate production by W3110 (wild type) and SZ40 (Δ*focA-pflB*::*FRT* Δ*frdBC*). Lactate production by W3110 and lactate production by SZ40 were compared by using pH-controlled fermentors and M9 medium containing 5% glucose as the sole carbon source (Fig. 2A and B, respectively). Glucose was not fully metabolized by W3110 in 192 h (Table 3). Even after 240 h of incubation, approximately 1% (50 mM) glucose remained. In contrast, SZ40 completed fermentation of 5% glucose within 192 h (Table 3). The lactate yields for SZ40 were more than twice those for W3110 and approached the theoretical maximum, two lactate molecules per glucose molecule (Table 3). Small but measurable amounts of ethanol and acetate were also produced by SZ40 during fermentation of 5% glucose (Table 3). Ethanol (7 mM) was the second most abundant fermentation product and represented less than 1% of the glucose carbon. Although the cell yield for SZ40 (363 mg/liter) was only 60% of the cell yield for the parent (601 mg/liter), the maximum volumetric and maximum specific productivities were higher for SZ40 (2.8- and 6.2-fold, respectively).

The kinetics of growth and lactate production were dramatically altered by the mutations in SZ40 (Fig. 2A and B). W3110 began to grow immediately upon inoculation and reached the maximal cell density within 48 h. With SZ40, an initial doubling of cell mass during the first 24 h was followed by a 2-day lag before growth resumed. The possibility that the resumption of growth after this lag resulted from secondary mutations in SZ40 was investigated in two ways. Cells from the later stage of SZ40 fermentation were serially transferred as an inoculum for a new fermentation. Also, clones were isolated from this culture and used to prepare seed cultures for new fermentations. With both approaches, the growth lag and unusual kinetics persisted, indicating that a mutant population of SZ40 was not responsible for the resumption of growth (data not shown).

Chiral purity of D-lactate. The purity of the D-lactate produced by SZ40 was determined by using the HPLC and a chiral column to separate lactate enantiomers (35). Based on these analyses, the SZ40 product was determined to contain less than 0.2% L-lactate.

Benefits of eliminating ethanol as a minor fermentation product. Although the amount of ethanol produced by SZ40 was quite small (Table 3; Fig. 3), reducing the level of this compound may significantly increase the lactate yield in large-scale fermentations. P1 transduction and FLP recombinase were used to construct an *adhE* deletion in SZ40. As expected, deletion of the *adhE* gene (SZ58) eliminated ethanol production (Fig. 3; Table 3). Two additional improvements were also noted when the mutant was compared to the parent, SZ40 (Fig. 2B and C): (i) there was a 36% increase in the maximum cell density (495 mg/liter, compared to 363 mg/liter for SZ40); and (ii) there was a small increase in lactate productivity. With SZ58, the time required to complete fermentation of 5% glucose was approximately 24 h less than the time required with SZ40. Strain SZ58 exhibited higher maximum volumetric and maximum specific productivities (114 and 128%, respectively) than SZ40. With both strains, the lactate yields remained near the theoretical maximum (two lactate molecules per glucose molecule). The differences in the reported values (99 versus 97%) are within the error of these measurements (Table 3).

The unusual growth pattern and lag observed for SZ40 persisted in SZ58 (Fig. 2B and C). Despite higher maxima for volumetric and specific productivities, the low level of biocat-

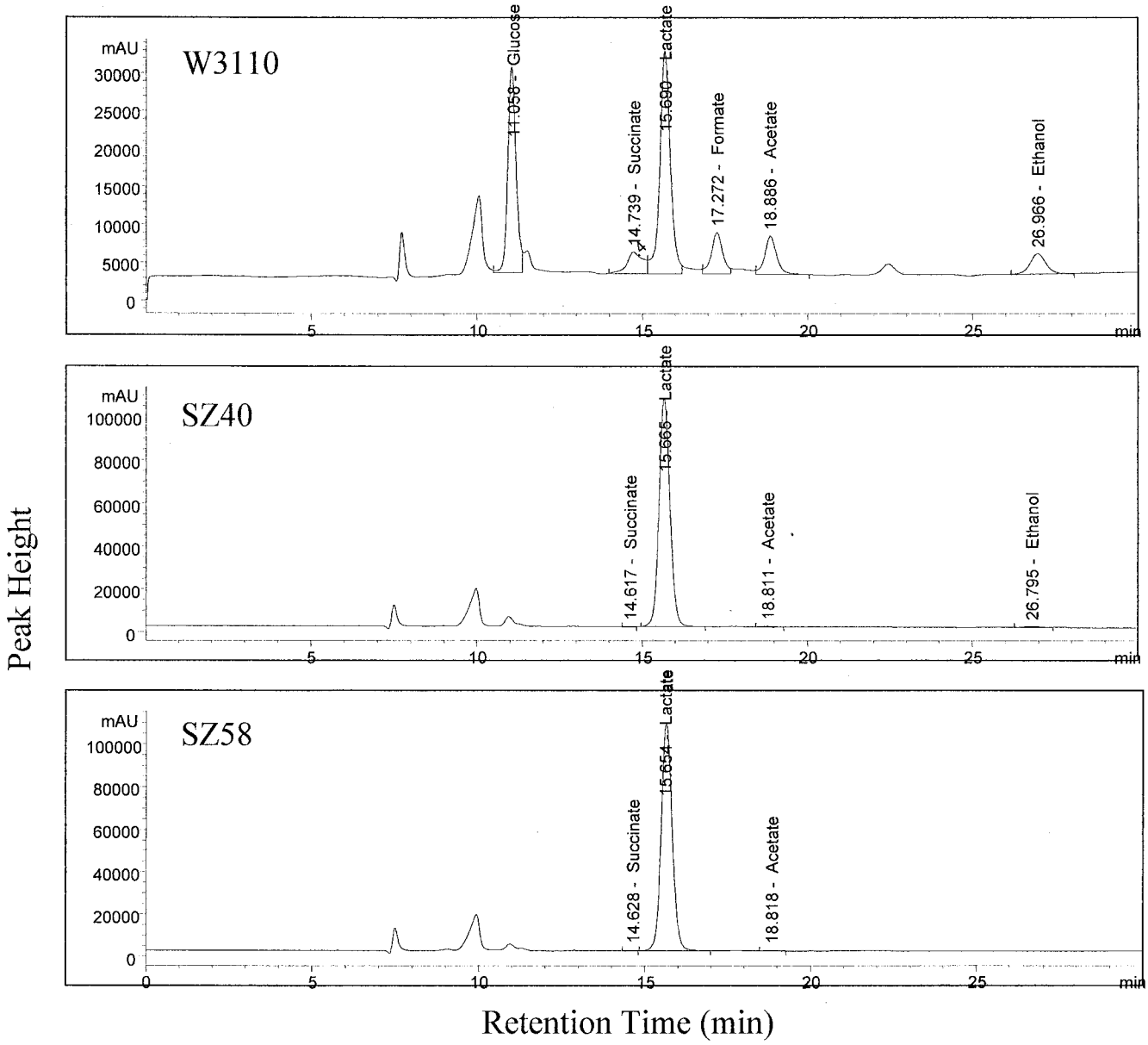

FIG. 3. HPLC profiles (refractive index monitor) from broth samples of W3110, SZ40, and SZ58 cultures at the end of fermentation. The two initial peaks that are not labeled are the inorganic components in M9 medium. The remaining peaks are identified by compound and retention time.

alyst during the first 96 h extended the time required to complete glucose fermentation. Two possible reasons for this lag were investigated. Sodium bicarbonate (1 g/liter) was added as a supplement to eliminate a potential deficiency of CO_2 (2); trace elements (30) were also added. Neither supplement was beneficial for growth or lactate production. The kinetics of fermentation and the lag in growth remained unchanged (data not shown).

Benefits of eliminating acetate as a minor fermentation product. Both SZ40 and SZ58 accumulated approximately 6 mM acetate in the broth during the initial 96 h of incubation, part of which was subsequently consumed during cell growth (Fig. 4). To reduce acetate production, an *ackA* mutant of SZ58 was constructed by using P1 transduction followed by treatment with FLP recombinase to excise the *tet* gene used for selection. Acetate production by the resulting strain (SZ63) was reduced by more than 80% during the initial 120 h of incubation but was not eliminated (Fig. 4).

Addition of the *ackA* mutation resulted in a dramatic and unexpected improvement in lactate productivity (Fig. 2C and D). The growth lags observed for SZ40 and SZ58 were absent in SZ63. Although the cell yield was lower for SZ63 than for SZ40 and SZ58 (and W3110), the rapid initial growth of SZ63 provided a higher level of biocatalyst early in the fermentation process, which in turn increased the rate of glucose conversion to lactate. The lactate levels for SZ63 after 72 and 96 h were more than twice those for SZ40 and SZ58 (Fig. 5). Like the lactate yields with SZ40 and SZ58, the lactate yield at the end

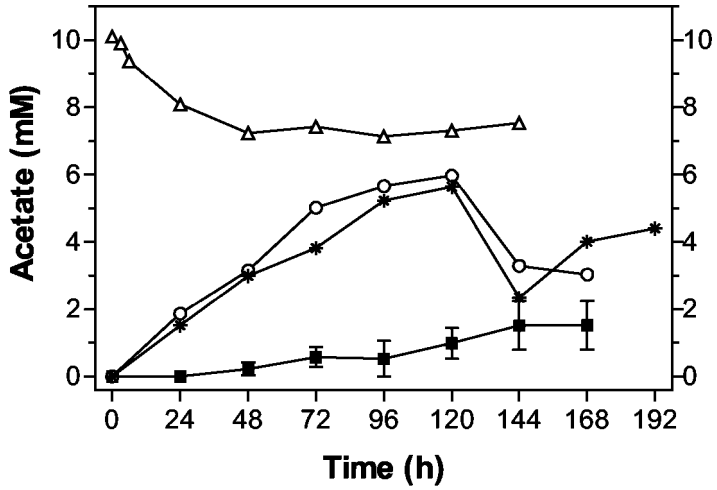

FIG. 4. Acetate production and utilization during fermentation. Symbols: ○, SZ58; △, SZ58 supplemented with 10 mM acetate; ∗, SZ40; ■, SZ63.

of SZ63 fermentations approached the theoretical maximum yield for glucose.

One immediate effect of an *ackA* mutation on metabolism is to increase the pools of acetyl-P and acetyl-CoA (9, 12, 27), providing a possible mode of action. Since both enzymes used for acetate production (phosphotransacetylase and acetate kinase) are known to be reversible (10, 23, 38), this hypothesis was tested by supplementing SZ58 and SZ63 fermentations with sodium acetate (10 mM). Addition of acetate to SZ58 eliminated the lag in growth (Fig. 2C and E) and stimulated lactate production (Fig. 5), but addition of acetate had no effect on SZ63 (data not shown). Addition of acetate to SZ58 increased the maximum volumetric productivity (9.1 mmol/liter per h) and the maximum specific productivity (44.6 mmol/g [dry weight] of cells per h). All growth occurred during the initial 48 h of incubation. During this period, the concentration of acetate declined in the broth (from 10 to 6.6 mM) and then remained essentially constant (Fig. 4). Presumably, the acetate was incorporated into SZ58 cell mass.

Process-based improvements in lactate production. Within the limitations of our measurements, the engineered biocatalysts catalyzed a one-for-one conversion of glucose carbon to lactic acid carbon, leaving little room for further improvement in yield or product purity (Table 3; Fig. 3). Conversion rates, however, are limited by the level of biocatalyst due to relatively slow growth and low cell yield (Fig. 2). Previous studies have shown (11) that an initial period of aeration in complex media can be used to boost the growth of D-lactate-producing strains of *E. coli* containing mutations in phosphoenolpyruvate carboxylase (*ppc*) and phosphotransacetylase (*pta*) genes and shorten the time required for fermentation. An initial period of aeration (8 h) was investigated with SZ63 and SZ58 in M9

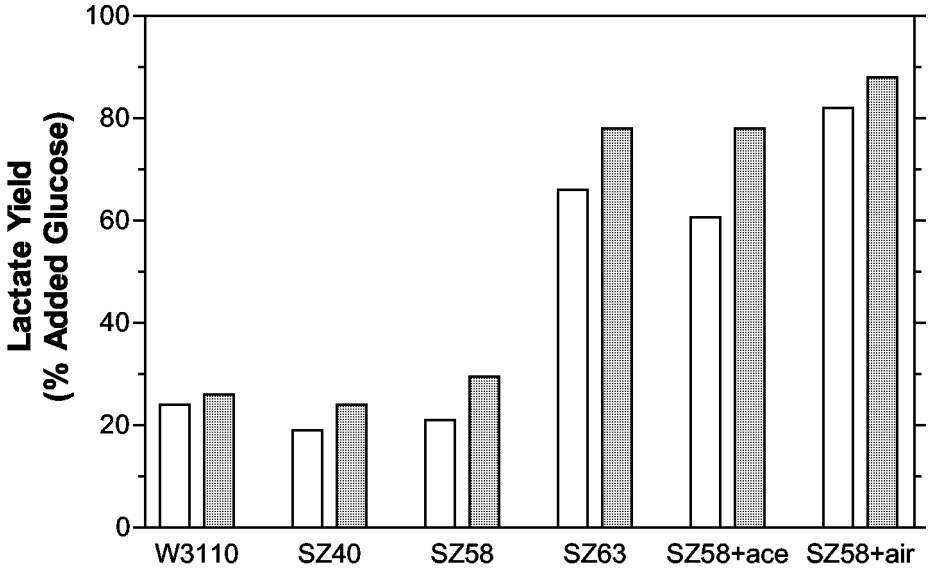

FIG. 5. Comparison of lactate production after 72 h (open bars) and 96 h (solid bars). The bacterial strains and additives (if present) are indicated at the bottom. ace, acetate.

medium containing 5% glucose (1% added initially and the balance added after 8 h). Oxygen levels were maintained at about 20% of air saturation by automatically mixing oxygen and nitrogen while a constant flow of 1 liter/min was maintained (400 rpm).

Initial aeration of an SZ58 culture eliminated the lag in growth and resulted in a 10-fold increase in cell yield within the first 24 h (Fig. 2C and F). This early increase in biocatalyst level accelerated glucose conversion to lactate (Fig. 5) and reduced the time required to complete fermentation of 5% glucose. Maximum volumetric productivity was essentially unchanged by aerobic growth despite the higher cell mass. Accordingly, the maximum specific productivity of cells from the initially aerated cultures was less than one-half that of cells grown anaerobically (after the lag or with acetate). The lactate yield, however, was reduced by initial aeration (Table 3). Unlike SZ40 and SZ58, strain SZ63 did not exhibit an initial growth lag. Growth and lactate production by SZ63 were not improved by an initial period of aeration (data not shown). While no attempt at further optimization was made, it is clear that changes in process conditions have the potential to reduce the time required to complete fermentations without the addition of complex nutrients.

DISCUSSION

The genetic engineering of prototrophic strains of *E. coli* (lacking plasmids and antibiotic resistance genes) as biocatalysts for the production of chemically pure D-lactic acid (~98% pure with respect to organic compounds) and optically pure D-lactic acid (>99% enantiomeric purity) has several advantages. The use of mineral salts medium should reduce costs associated with ingredients, product purification, biological oxygen demand reduction, and waste treatment. Native lactic acid bacteria typically require complex nutrients for growth (24) and seldom achieve the enantiomeric purity or product selectivity obtained with SZ40 and its derivatives. The choice of the genes used to inactivate competing fermentation pathways is critical to maintain the yield and to minimize the nutritional requirements. Use of a *ppc* mutation to reduce succinate production in a previous study (22, 43) severely limited biosynthesis of the aspartate family of amino acids and other cellular constituents, creating an auxotrophic requirement which could be met only by amino acids or intermediates of the tricarboxylic acid pathway (23, 43). In our biocatalysts (SZ40, SZ58, and SZ63), major biosynthetic pathways were left intact by deleting genes encoding two subunits of the fermentative fumarate reductase (Δ*frdBC*). The requirements for succinate can readily be met by alternative pathways, such as the glyoxylate cycle.

The cell yields for SZ40, SZ58, and SZ63 remained less than 1 g/liter during anaerobic growth in M9 medium, equivalent to less than 2% of the sugar metabolized. Although metabolic activity can be high (44.6 mmol of lactate/g [dry weight] of cells per h; approximately 1.35 μmol/mg of cell protein per min), the low concentrations of biocatalysts in these fermentations restrict the volumetric rate of lactate production. The low cell yield (low concentration of biocatalyst) was further exacerbated by the delayed growth of SZ40 and SZ58 (Fig. 2B and C). This growth lag was attributed to a problem in carbon partitioning, insufficient acetyl-CoA, or acetyl-P. Although small amounts of acetate were present in the broth of SZ40 and SZ58 at the end of fermentation (Table 3), which is consistent with an excess of acetyl-CoA, a more detailed analysis of HPLC data during fermentation indicated that there was a possible relationship between the growth of SZ40 and SZ58 and assimilation of acetate from the broth (Fig. 4). Prior to the resumption of growth, acetate accumulated slowly to a maximum concentration of about 6 mM, approximately 4 mM of which was used during the late burst of growth (Fig. 2B and C; Fig. 4). Adding 10 mM acetate eliminated the growth lag in SZ58, and one-third of the acetate was used during the initial growth of this strain. The decrease in the amount of acetate during growth (201 mg/liter) was approximately equal to one-half of the dry cell weight of SZ58 (405.9 mg/liter) in acetate-supplemented fermentations. Thus, the accumulation of acetate in the broth cultures of SZ40 and SZ58 appeared to be responsible for the resumption in growth after 96 h. It is interesting that even higher levels of acetate (20 mM) were produced in the broth of SZ58 cultures during initial aeration concurrent with rapid growth (Fig. 2F). Inactivation of *ackA* (SZ63) eliminated the growth lag by blocking the drain of acetyl-CoA into acetate. This mutation reduced the net acetate accumulation in the broth by up to 80% (Fig. 4). The accumulation of acetate during the growth lag (SZ40 and SZ58), the beneficial effects of supplementing SZ58 cultures with acetate, and the *ackA* mutation in SZ63 together provide evidence that the availability of acetyl-CoA (or acetyl-P) limits the initial growth of SZ40 and SZ58 during homolactate fermentation. The final cell yield was improved by addition of an *adhE* mutation (SZ58), declined with further addition of an *ackA* mutation (SZ63), and was not improved by addition of 10 mM acetate. The cell yield was doubled (SZ58), however, by an initial period of aeration, demonstrating the nutritional adequacy of M9 medium with glucose. Factors other than nutrient limitation must limit cell yield (Table 3).

The stimulation of growth by aeration observed with SZ58 was eliminated by introduction of an *ackA* mutation (SZ63), a mutation which was beneficial for anaerobic growth. Numerous studies have previously shown that mutations in the acetate pathway (*pta*, *ackA*) reduce the growth of *E. coli* under aerobic conditions (12, 15). Although the basis remains unknown, problems associated with the turnover of acetyl-CoA (or acetyl-P) and other metabolic imbalances have been implicated (9, 12, 27). Addition of a recombinant pathway for polyhydroxybutyrate biosynthesis from acetyl-CoA relieved the detrimental effect of *ackA* during aerobic growth (12).

The lag in growth and the resultant decrease in lactate production observed with SZ40 and SZ58 are undesirable traits that increase the time required to complete fermentation. Elimination of the lag by initial aeration suggests that further process optimization may increase lactate productivity while preserving high yields. The high D-lactate yields and chiral purity obtained with SZ40, SZ58, and SZ63 are equal to or better than the D-lactate yields and chiral purity previously reported for other biocatalysts (11, 17, 24).

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