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# Effects of Eliminating Pyruvate Node Pathways and of Coexpression of Heterogeneous Carboxylation Enzymes on Succinate Production by *Enterobacter aerogenes*

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**Lowering the pH in bacterium-based succinate fermentation is considered a feasible approach to reduce total production costs. Newly isolated *Enterobacter aerogenes* strain AJ110637, a rapid carbon source assimilator under weakly acidic (pH 5.0) conditions, was selected as a platform for succinate production. Our previous work showed that the Δ*adhE*/PCK strain, developed from AJ110637 with inactivated ethanol dehydrogenase and introduced *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase (PCK), generated succinate as a major product of anaerobic mixed-acid fermentation from glucose under weakly acidic conditions (pH <6.2). To further improve the production of succinate by the Δ*adhE*/PCK strain, metabolically engineered strains were designed based on the elimination of pathways that produced undesirable products and the introduction of two carboxylation pathways from phosphoenolpyruvate and pyruvate to oxaloacetate. The highest production of succinate was observed with strain ES04/PCK+PYC, which had inactivated ethanol, lactate, acetate, and 2,3-butanediol pathways and coexpressed PCK and *Corynebacterium glutamicum* pyruvate carboxylase (PYC). This strain produced succinate from glucose with over 70% yield (gram per gram) without any measurable formation of ethanol, lactate, or 2,3-butanediol under weakly acidic conditions. The impact of lowering the pH from 7.0 to 5.5 on succinate production in this strain was evaluated under pH-controlled batch culture conditions and showed that the lower pH decreased the succinate titer but increased its yield. These findings can be applied to identify additional engineering targets to increase succinate production.**

There is an increasing interest in bio-based chemicals from renewable carbon sources because of the increasing price of petroleum and the negative impact of petrochemical production on the environment (1, 2). Succinate, a $C_4$-dicarboxylic acid, which is an intermediate metabolite in the tricarboxylic acid cycle, is potentially useful as a chemical precursor for many commodity chemicals, such as γ-butyrolactone, tetrahydrofuran, and 1,4-butanediol. These chemicals can, in turn, be converted into a wide variety of products, such as green solvents, pharmaceuticals, and biodegradable plastics (3, 4). Lowering the pH of microbial cultures has been considered a feasible approach to reducing the total costs of succinate production by limiting the use of alkali and acids in the fermentation and recovery processes (5, 6). Although anaerobic succinate production by *Escherichia coli*, *Corynebacterium glutamicum*, *Actinobacillus succinogenes*, and *Anaerobiospirillum succiniciproducens* has been studied with pHs ranging from 6.0 to 7.0 (7–9), few studies have focused on the effect of weakly acidic pH (pH <6.0) on succinate production by bacteria. This is because these bacteria are sensitive to acidic stress and are unable to grow and assimilate carbon sources effectively under weakly acidic conditions (10, 11). One potential solution to this limitation is to develop a new platform for producing succinate by using bacteria that are inherently adapted to acidic conditions (12). Such studies have the potential to advance bacterium-based succinate production processes.

*Enterobacter aerogenes* can rapidly assimilate carbon sources, such as glucose and glycerol, under moderately acidic conditions (pH <6.0), and it effectively produces biofuels, such as 2,3-butanediol, hydrogen, and ethanol, under anaerobic conditions (13–16). Recently, the whole genome sequence of *E. aerogenes* KCTC2190 was determined (17). Thus, the anaerobic central pathways involved in ethanol, lactate, 2,3-butanediol, and succinate formation can be predicted. The availability of this information provided the incentive to evaluate the suitability of this organism as a platform for succinate production under weakly acidic and anaerobic conditions.

A newly isolated *E. aerogenes* strain, AJ110637, that more rapidly consumed glucose under anaerobic conditions (pH 5.0) than did the well-characterized ATCC 13048 strain was selected as the platform strain. This strain was used to construct the Δ*adhE*/PCK strain, which has inactivated alcohol dehydrogenase (ADH) and introduced *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase (PCK). The Δ*adhE*/PCK strain produced succinate from glucose with a 60% yield under weakly acidic (pH <6.2) and anaerobic conditions (18). Compared with previously constructed *E. coli* succinate producers, such as KJ122 and KJ134, whose yields are near theoretical (19), the succinate titer and yield

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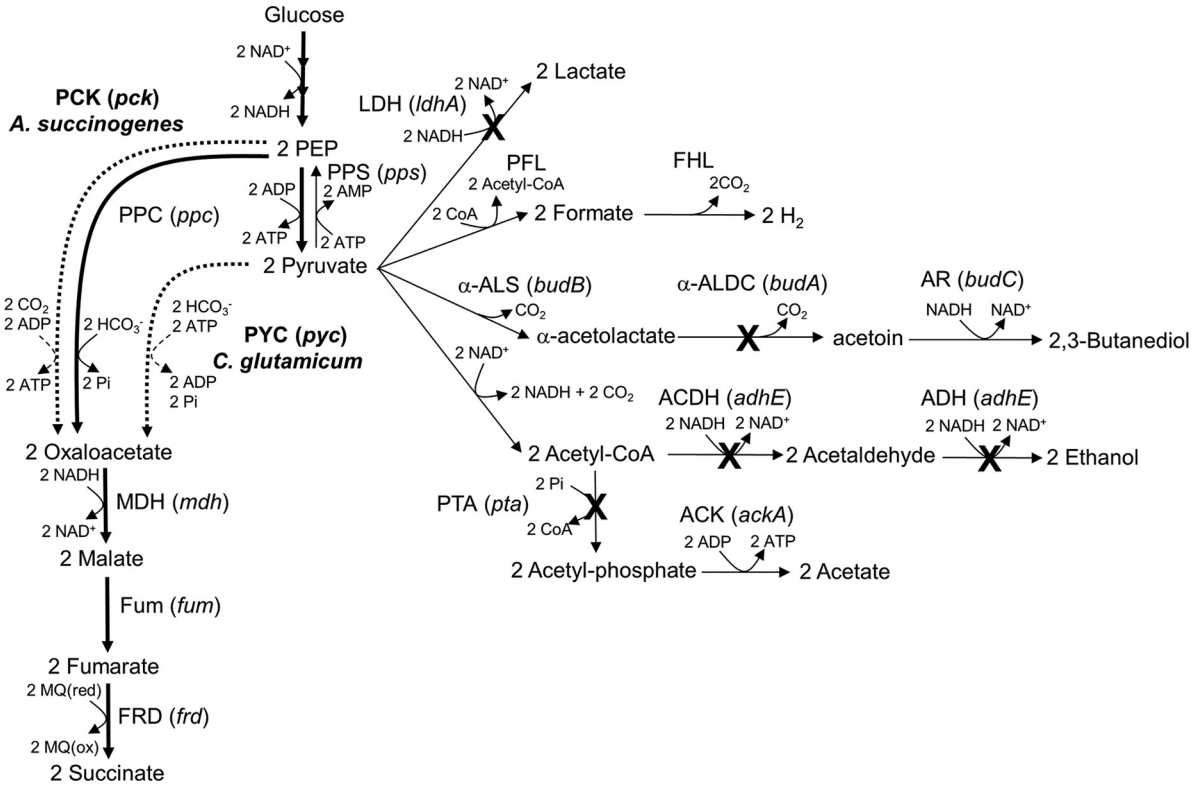


**FIG 1** Pathways involved in ethanol, 2,3-butanediol, lactate, acetate, and formate (thin arrows), as well as succinate synthesis (thick arrows), in *E. aerogenes*. Broken arrows indicate exogenous pyruvate carboxylase and PEP carboxykinase from *C. glutamicum* and *A. succinogenes*, respectively. The gene names or locus symbols are shown in parentheses. PCK, PEP carboxykinase; PYC, pyruvate carboxylase; MDH, malate dehydrogenase; Fum, fumarase; FRD, fumarate reductase; LDH, D-lactate dehydrogenase; PFL, pyruvate formate-lyase; FHL, formate hydrogen lyase; α-ALS, α-acetolactate synthase; α-ALDC, α-acetolactate decarboxylase; AR, acetoin reductase; ACDH, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase; PTA, phosphate acetyltransferase; ACK, acetate kinase; PPS, PEP synthetase.

from the Δ*adhE*/PCK strain is lower and needs to be improved with further metabolic engineering applications.

One metabolic modification often used to improve succinate production in various bacteria is the elimination of pathways that compete with succinate synthesis and yield undesirable products (19–21). Differences in anaerobic metabolism between *E. aerogenes* and well-studied succinate producers, such as *E. coli* and *C. glutamicum*, indicate that the target pathways that should be inactivated for improvement of succinate production in *E. aerogenes* are different from those in *E. coli* and *C. glutamicum* (22–25). An optimal strategy for improvement of succinate production by *E. aerogenes* remains to be determined but is important to better understand the anaerobic metabolism of this organism.

A general strategy to increase succinate synthesis is to enhance the carboxylation pathways (26–28). In particular, the introduction of two carboxylation pathways from phosphoenolpyruvate (PEP) and pyruvate to oxaloacetate (OAA) effectively stimulates succinate production (Fig. 1). For example, coexpression of *Sorghum vulgare* PEP carboxylase (PPC) and *Lactococcus lactis* pyruvate carboxylase (PYC) in *E. coli* increased succinate production to a greater extent than expression of either pathway alone (29). In the present study, based on these strategies, we generated strain ES04/PCK+PYC, with genes *adhE*, *ldhA*, *pta*, and *budA* deleted and with a new coexpression system involving *A. succinogenes* PCK and *C. glutamicum* PYC introduced. This strain produced succinate from glucose with over 70% yield without the formation (i.e., below 0.1 g/liter) of ethanol, lactate, or 2,3-butadiol under a weakly acidic condition. This strain was also used to investigate the impact on succinate production of lowering the pH from 7.0 to 5.5.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Details of the strains and plasmids used in this study are summarized in Table 1. *E. aerogenes* AJ110637 was deposited at the International Patent Organism Depository, Agency of Industrial Science and Technology (Japan), under accession no. FERM P-21348 (45). The deposit was converted to an international deposit and assigned receipt no. FERM BP-10955 (18). Plasmids were introduced into *E. coli* and *E. aerogenes* by electrotransformation. Both *E. coli* and *E. aerogenes* were grown in Luria-Bertani (LB) medium at 37°C. When needed, 50 mg/liter kanamycin or 40 mg/liter chloramphenicol was added to select transformants and to maintain the plasmids.

**Disruption of *ldhA*, *pta*, and *budA* genes.** To disrupt the *ldhA*, *pta*, and *budA* genes, the λ Red gene knockout system was used with the Red-recombineering helper plasmid pRSFRedTER (18, 30, 31) (see Fig. S1A in the supplemental material). A removable kanamycin resistance gene flanked by $attL_\lambda$ and $attR_\lambda$ was amplified with Δ*ldhA-attL*/Δ*ldhA-attR*, Δ*pta-attL*/Δ*pta-attR*, and Δ*budA-attL*/Δ*budA-attR* primers containing 60-nucleotide (nt) sequences homologous to the target region at the 5′ end of the chromosome. The pMW-$attL_\lambda$-Km$^r$-$attR_\lambda$ plasmid was used as the DNA template (30, 31). Replacement of the target genes on the chromosome with the $attL_\lambda$-Km$^r$-$attR_\lambda$ fragment was confirmed by PCR using Δ*ldhA*-CF/Δ*ldhA*-CR, Δ*pta*-CF/Δ*pta*-CR, and Δ*budA*-CF/Δ*budA*-CR as primers. To remove pRSFRedTER from the marker strains, the strains

**TABLE 1** Microbial strains and plasmids used in this study

| Strains or plasmid | Description | Antibiotic resistance[a] | Reference or source |
|---|---|---|---|
| Strains | | | |
| AJ110637 | Isolated wild-type strain (FERM BP-10955) | | 18 |
| Δ*adhE* mutant | AJ110637 Δ*adhE* | Km | 18 |
| ES02 | AJ110637 Δ*adhE* Δ*ldhA* | Km | This work |
| ES03 | AJ110637 Δ*adhE* Δ*ldhA* Δ*pta* | Km | This work |
| ES04 | AJ110637 Δ*adhE* Δ*ldhA* Δ*pta* Δ*budA* | Km | This work |
| Plasmids | | | |
| pKD46 | λ Red-expressing plasmid | Ap | 30 |
| pRSFRedTER | Broad-host-range λ Red-expressing plasmid | Cm | 31 |
| pMW-*attL*$_\lambda$-Km$^r$-*attR*$_\lambda$ | Cassette for gene disruption containing kanamycin resistance gene | Km | 31 |
| pMW-intxis-ts | λ Int/Xis-expressing plasmid | Ap | 32, 33 |
| pRSF-*P*$_{ara}$-IX | Broad-host-range λ Int/Xis-expressing plasmid | Cm | This work |
| pSTV28 | Plasmid vector with a replication origin of pACYC184 | Cm | TaKaRa BIO |
| pSTV28-*pyc* | Plasmid for expression of *pyc* from *C. glutamicum* | Cm | 18 |
| pSTV28-*pck* | Plasmid for expression of *pck* from *A. succinogenes* | Cm | 18 |
| pSTV28-*pck*-*pyc* | Plasmid for coexpression of *A. succinogenes pck* and *C. glutamicum pyc* | Cm | This work |

[a] Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol.

were spread on LB plates containing 10% sucrose and 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to obtain single colonies. Removal of pRSFRedTER was confirmed on LB plates containing 40 mg/liter chloramphenicol. The strain that could not grow under these conditions was designated the "pRSFRedTER-free" strain. All primer sequences are listed in Table 2.

**Construction of pRSF-*P*$_{ara}$-IX.** pRSF-*P*$_{ara}$-IX was constructed to remove the kanamycin resistance gene on the chromosome in the marker strains. The pMW-intxis-ts plasmid carries the gene encoding integrase (Int) of the λ phage and the gene encoding excisionase (Xis) and provides temperature-sensitive replication (32, 33). The pKD46 plasmid carries the gene encoding the λ Red genes under the control of an arabinose promoter (30). DNA fragments containing the *xis-int* and arabinose promoter regions were amplified by PCR using the intxis-F/intxis-R and PKD466199F/PKD461243R primers and pMW-intxis-ts and pKD46 as the templates, respectively. The *xis-int* and arabinose promoter regions were cloned into the NotI/PvuI and BsaI/PvuI sites of the pRSFRedTER plasmid, respectively. This plasmid was designated "pRSF-*P*$_{ara}$-IX" (see Fig. S1B in the supplemental material).

**Removal of antibiotic resistance genes from the chromosome.** pRSF-*P*$_{ara}$-IX was used to remove the kanamycin resistance gene from the genetically modified bacterial chromosome (see Fig. S1 in the supplemental material). pRSF-*P*$_{ara}$-IX was introduced into the marker strain via electrotransformation. Transformants harboring pRSF-*P*$_{ara}$-IX were se-

**TABLE 2** Oligonucleotide primer sequences used in this study

| Primer | Primer sequence (5′→3′) |
|---|---|
| Disruption of *adhE*, *budA*, *ldhA*, and *pta* genes | |
| Δ*budA-attL* | 5′-AACCTTTATTTAACCTTTCTTATATTTGTTGAACGAGGAAGTGGCTCATGTGAAGCCTGCTTTTTTATACTAAGTTGGC-3′ |
| Δ*budA-attR* | 5′-GCGCCCACTGGCGCTGCGGATACTGTTTGTCCATGTGAACCTCCTAACTTCGCTCAAGTTAGTATAAAAAAGCTGAACGA-3′ |
| Δ*ldhA-attL* | 5′-ATGTTAACGACGCATACGGCTTTGAACTGGAATTTTTCGACTTCCTGCTGACCGAAAAGATGAAGCCTGCTTTTTTATACTAAGTTGGC-3′ |
| Δ*ldhA-attR* | 5′-GGCAGGCGGAGAGCCGGCGGAACACGTCATCCTGGATGACATCATTCGATTTATCTTCAACGCTCAAGTTAGTATAAAAAAGCTGAACGA-3′ |
| Δ*pta-attL* | 5′-CTGGCGGTGCTGTTTTGTATCCCGCCTAAAACTGGCGGTAACGAAAGAGGATATATCGTGTGAAGCCTGCTTTTTTATACTAAGTTGGC-3′ |
| Δ*pta-attR* | 5′-GCGTTAGAGCCATAAAAAAGGCAGCCATTTGGCTGCCTTTCTTGTCTCAGCGGGAGATTACGCTCAAGTTAGTATAAAAAAGCTGAACGA-3′ |
| Δ*budA*-CF | 5′-TTTCTATATTGGAACTGTGAGCTGAATCG-3′ |
| Δ*budA*-CR | 5′-GCTTCCAGTTGGCTGACGACCAGATC-3′ |
| Δ*ldhA*-CF | 5′-TTTCTTAAGACTGCGATATGCTCTAG-3′ |
| Δ*ldhA*-CR | 5′-TTCATTCGTACTCCAAAACATTTGTC-3′ |
| Δ*pta*-CF | 5′-TCGTGAACTGTCCCTGGG-3′ |
| Δ*pta*-CR | 5′-GTCAGCGAGGTATGCTGG-3′ |
| Construction of pRSF-*P*$_{ara}$-IX | |
| intxis-F | 5′-CATGGCGGCCGCTTATTTGATTTCAATTTTGTCCCAC-3′ |
| intxis-R | 5′-GATCCCGATCGAAGGAGGTTATAAAAAATGGAATTGAATTCGTGTAATTGC-3′ |
| PKD466199F | 5′-GCGCGGTCTCACTCTTCCTTTTTCAATA-3′ |
| PKD461243R | 5′-GGCCCGATCGTTTTTATAACCTCCTTAG-3′ |

lected on LB plates containing 40 mg/liter of chloramphenicol. Obtained transformants were spread on LB plates containing 40 mg/liter chloramphenicol and 1% L-arabinose. Clones that formed single colonies were identified, and elimination of the kanamycin resistance gene was confirmed by growth on LB medium containing 50 mg/liter kanamycin. Strains that could not grow on the kanamycin plates were utilized as marker-free strains. The procedure to remove pRSF-$P_{ara}$-IX from the marker-free strain was the same as that of pRSFRedTER (18).

**Construction of plasmid to co-overexpress *A. succinogenes* PCK and *C. glutamicum* PYC.** DNA fragments obtained by SacI digestion, containing the *thrL* promoter and *C. glutamicum pyc* of pSTV28-*pyc*, were ligated into SacI-digested pSTV28-*pck* in order to obtain a plasmid that coexpressed PCK and PYC. The resultant plasmid was designated "pSTV28-*pck*-*pyc*." The strains harboring pSTV28, pSTV28-*pck*, pSTV28-*pyc*, and pSTV28-*pck*-*pyc* are formatted as follows: "strain name/empty," "strain name/PCK," "strain name/PYC," and "strain name/PCK+PYC," respectively (e.g., the Δ*adhE* mutant harboring pSTV28-*pck* is represented as the "Δ*adhE*/PCK" strain).

**Succinate fermentation in 1.5-ml microcentrifuge tubes.** To evaluate succinate production under weakly acidic (pH <6.2) and anaerobic conditions, we employed a convenient evaluation system utilizing 1.5-ml microcentrifuge tubes (18). Preculture for cell growth was performed on LB plates at 37°C for 10 h. The precultured cells were then incubated anaerobically at 37°C for 16 h in an AnaeroPack A-04 (Mitsubishi Gas Chemicals, Inc., Tokyo, Japan), washed twice with cold NaCl solution (0.8%), and inoculated into 1.5-ml microcentrifuge tubes containing 1.4 ml MS medium [40 g/liter glucose, 1 g/liter $MgSO_4 \cdot 7H_2O$, 2 g/liter Bacto yeast extract, 1 g/liter $(NH_4)_2SO_4 \cdot 7H_2O$, 1 g/liter $KH_2PO_4$, 10 mg/liter $MnSO_4 \cdot 5H_2O$, 10 mg/liter $FeSO_4 \cdot 7H_2O$, 1 mg/liter biotin] containing 50 g/liter precipitated $CaCO_3$ sterilized by dry heat at 180°C for 3 h (Japanese Pharmacopoeia, Tokyo, Japan). After tight capping, succinate fermentation was performed using an Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany) at 34°C and a rotation speed of 1,400 rpm. The initial biomass was adjusted to approximately 1.2 g dry cell weight (DCW) per liter. Using this system, the pH dropped from an initial 6.2 to approximately 5.4 due to the formation of acidic compounds, such as succinate, during fermentation.

**pH-controlled succinate fermentation.** pH-controlled succinate fermentation was carried out in a 100-ml jar fermenter with 60 ml MS2 medium [50 g/liter glucose, 1 g/liter $MgSO_4 \cdot 7H_2O$, 2 g/liter Bacto yeast extract, 1 g/liter $(NH_4)_2SO_4 \cdot 7H_2O$, 1 g/liter $KH_2PO_4$, 10 mg/liter $MnSO_4 \cdot 5H_2O$, 10 mg/liter $FeSO_4 \cdot 7H_2O$, 1 mg/liter biotin, 0.05 g GD113 (antifoam reagent)]. The initial pH was adjusted to 5.5, 5.7, or 7.0 by KOH at 34°C. Each pH was maintained with 2 N NaOH during cultivation. Carbon dioxide gas was applied at 40 ml/min, and agitation was set at 700 rpm. Preculture was performed on LB agar at 37°C for 10 h. The cells were then incubated at 37°C for 16 h in an AnaeroPack A-04. Initial biomass was adjusted to approximately 1.2 g (DCW)/liter.

**Analysis of metabolites.** Organic acids that accumulated in the medium were analyzed by high-performance liquid chromatography on a CDD-10AD system (Shimadzu Co., Ltd., Kyoto, Japan) after a suitable dilution (34). Acetoin, 2,3-butanediol, and ethanol were quantified on a GC4000 gas chromatograph (GL-Science Co., Ltd., Tokyo, Japan) equipped with a flame ionization detector. A TC-BOND Q (GL-Science) capillary column was used for separation (18). The glucose concentration was analyzed using an AS-310 Biotech analyzer (Sakura SI Co., Ltd., Tokyo, Japan). Yield is expressed as gram of product per gram of consumed glucose (g/g). Optical densities at 600 nm ($OD_{600}$) were measured with a U-2001 spectrometer (Hitachi Co. Ltd., Tokyo, Japan). Broth containing $CaCO_3$ was diluted with 0.1 N HCl before measurement of $OD_{600}$. Dry cell weight was calculated according to a standard curve between the g (DCW)/liter and $OD_{600}$. The formula used to calculate the dry cell weight of *E. aerogenes* was g (DCW)/liter = 0.291 × $OD_{600}$.

## RESULTS

**Development of a λ Int/Xis-dependent selective marker excision system in *E. aerogenes*.** The λ Red-recombination system has been successfully used to rapidly generate chromosomal modifications in members of the *Enterobacteriaceae* family (14, 30, 31). In our previous study, we demonstrated λ Red-dependent integration using pRSFRedTER to disrupt the *adhE* gene in *E. aerogenes* (Table 1; see Fig. S1A in the supplemental material). To accelerate genome engineering, we modified a selective marker excision system for *E. aerogenes*. A λ Int/Xis-dependent site-specific recombination system is available to remove selective markers from the *E. coli* chromosome (see Fig. S1A) (32, 33). In this system, the pMW-intxis-ts plasmid contains β-lactamase as a selective marker and is used for the expression of λ Int/Xis protein in *E. coli* (Table 1). However, *E. aerogenes* exhibits inherently strong resistance toward ampicillin, and there are multiple copies of the β-lactamase gene on the *E. aerogenes* KCTC2190 chromosome. For this reason, we reconstructed a λ Int/Xis expression plasmid from pRSFRedTER that contains a different selection marker, chloramphenicol acetyltransferase (encoded by *cat*) and levansucrase (encoded by *sacB*) from *Bacillus subtilis*. This allows for efficient recovery of this plasmid from cells grown in a sucrose-containing medium (31). A newly constructed λ Int/Xis expression plasmid (pRSF-$P_{ara}$-IX) provided L-arabinose-inducible expression of the *xis* and *int* genes under the control of an arabinose promoter (Fig. S1B). A marker-free strain was obtained from the Δ*adhE* ($Km^r$) strain harboring pRSF-$P_{ara}$-IX after it was cultured in LB medium containing 1% L-arabinose (details may be found in Materials and Methods).

**Effect of inactivation of lactate dehydrogenase on succinate production in the Δ*adhE* mutant.** Our previous study showed that lactate was a significant by-product in the Δ*adhE*/PYC and Δ*adhE*/PCK strains, which produced 7.0 and 1.6 g/liter lactate with yields of 45.5% and 18.6% (g/g), respectively (18). Lactate is formed from pyruvate by coupling with the oxidation of NADH. D-Lactate dehydrogenase (LDH) (encoded by *ldhA*) is the dominant catalyst for this reaction in *E. aerogenes* (14) (Fig. 1). The *ldhA* gene was, therefore, disrupted from the Δ*adhE* mutant (designated ES02) to reduce lactate formation (Table 1). Then, the heterogeneous carboxylation enzyme expression plasmids, pSTV28-*pyc* and pSTV28-*pck*, were introduced into ES02 to enhance succinate production (Table 1). The results of succinate fermentation in 1.5-ml microcentrifuge tubes using these strains are summarized in Table 3. Lactate formation was not observed in ES02/empty. However, the acetate titer and yield were increased in ES02/empty (1.8 g/liter corresponding to a 52.9% yield) compared to the Δ*adhE*/empty strain (0.5 g/liter corresponding to a 20.8% yield). The succinate titers in strains ES02/PYC and ES02/PCK were 4.8 g/liter and 4.6 g/liter, respectively. These values were approximately 0.9 times of those in the Δ*adhE*/PYC (5.4 g/liter) and Δ*adhE*/PCK (5.2 g/liter) strains (Table 3) (18). The succinate yield in ES02/PYC (51.6%) was increased compared with that in the Δ*adhE*/PYC strain (35.0%). In contrast, the succinate yield in ES02/PCK (63.9%) was similar to that in the Δ*adhE*/PCK strain (60.5%). Both ES02/PYC and ES02/PCK produced 3.3 g/liter acetate as a major by-product. The yields of acetate in ES02/PYC (35.5%) and ES02/PCK (45.8%) were 1.9 and 1.8 times those in the Δ*adhE*/PYC (18.8%) and Δ*adhE*/PCK (25.6%) strains, respectively. Thus, elimination of lactate formation in ES02/PYC and

**TABLE 3** Profiles of end pH, biomass, consumed glucose, and end products in multiple knockout mutants overexpressing PYC and/or PCK[a]

| Strain[b] | End pH | Biomass (g [DCW]/liter)[c] | Consumed glucose (g/liter) | End product (g/liter) | | | | | | | | Succinate yield (% [g/g])[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Pyruvate | Malate | Formate | Succinate | Lactate | Acetate | Ethanol | 2,3-Butanediol | |
| ES02/empty | 5.8 ± 0.1 | 1.2 ± 0.1 | 3.4 ± 0.4 | 0.4 ± 0.1 | ND[e] | 0.7 ± 0.1 | 0.2 ± 0.1 | ND | 1.8 ± 0.1 | ND | 0.2 ± 0.1 | 5.9 ± 1.1 |
| ES02/PYC | 5.4 ± 0.1 | 1.6 ± 0.1 | 9.3 ± 0.2 | ND | ND | 0.2 ± 0.1 | 4.8 ± 0.1 | ND | 3.3 ± 0.1 | ND | 0.2 ± 0.1 | 51.6 ± 0.9 |
| ES02/PCK | 5.4 ± 0.1 | 1.5 ± 0.1 | 7.2 ± 0.4 | <0.1 | <0.1 | <0.1 | 4.6 ± 0.3 | <0.1 | 3.3 ± 0.1 | ND | 0.2 ± 0.1 | 63.9 ± 3.1 |
| ES03/empty | 5.7 ± 0.1 | 1.3 ± 0.1 | 3.7 ± 0.3 | 0.5 ± 0.1 | ND | 0.3 ± 0.1 | 0.3 ± 0.1 | ND | 0.5 ± 0.2 | <0.1 | 1.4 ± 0.2 | 9.1 ± 2.9 |
| ES03/PYC | 5.5 ± 0.1 | 1.5 ± 0.1 | 8.9 ± 0.5 | ND | ND | <0.1 | 3.8 ± 0.2 | <0.1 | 0.4 ± 0.1 | <0.1 | 3.1 ± 0.2 | 42.7 ± 2.5 |
| ES03/PCK | 5.4 ± 0.1 | 1.5 ± 0.2 | 7.4 ± 0.7 | 0.2 ± 0.1 | ND | 0.2 ± 0.1 | 4.2 ± 0.2 | ND | 0.5 ± 0.2 | <0.1 | 3.7 ± 0.2 | 56.8 ± 4.4 |
| ES04/empty | 5.7 ± 0.1 | 1.4 ± 0.1 | 3.1 ± 0.2 | 0.8 ± 0.1 | ND | 0.4 ± 0.1 | 0.5 ± 0.2 | ND | 0.8 ± 0.1 | <0.1 | <0.1 | 15.1 ± 2.8 |
| ES04/PYC | 5.5 ± 0.1 | 1.6 ± 0.1 | 6.2 ± 0.3 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 3.6 ± 0.3 | <0.1 | 0.4 ± 0.1 | <0.1 | <0.1 | 58.1 ± 4.2 |
| ES04/PCK | 5.5 ± 0.1 | 1.5 ± 0.1 | 5.8 ± 0.3 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 3.5 ± 0.3 | ND | 0.5 ± 0.1 | <0.1 | <0.1 | 60.3 ± 3.6 |
| ES02/PCK+PYC | 5.4 ± 0.1 | 1.5 ± 0.1 | 8.5 ± 0.4 | <0.1 | 0.2 ± 0.1 | <0.1 | 5.7 ± 0.3 | ND | 3.1 ± 0.1 | ND | 0.2 ± 0.1 | 67.0 ± 4.3 |
| ES03/PCK+PYC | 5.4 ± 0.1 | 1.5 ± 0.2 | 9.7 ± 0.9 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 5.9 ± 0.2 | ND | 0.7 ± 0.2 | <0.1 | 3.4 ± 0.2 | 60.8 ± 4.8 |
| ES04/PCK+PYC | 5.4 ± 0.1 | 1.6 ± 0.1 | 11.4 ± 0.9 | 0.3 ± 0.1 | 0.5 ± 0.1 | <0.1 | 8.1 ± 0.4 | <0.1 | 0.6 ± 0.1 | <0.1 | <0.1 | 71.0 ± 2.8 |

[a] Data are expressed as means ± SD from four independent experiments.
[b] All strains were cultivated for 48 h.
[c] Initial biomass was adjusted to 1.2 g (DCW/liter).
[d] Succinate yield is gram of product per gram of consumed glucose expressed as a percentage.
[e] ND, not determined.

ES02/PCK leads to a slightly decreased succinate titer and enhanced acetate production.

**Effect of inactivation of PTA on succinate production in ES02.** Acetate was a significant by-product in strains ES02/PYC and ES02/PCK. Therefore, we next disrupted the *pta* gene in strain ES02 to reduce acetate production. The *pta* gene codes for phosphoacetyl transferase (PTA), which catalyzes the reversible conversion of acetyl coenzyme A (acetyl-CoA) to acetylphosphate in *E. coli* (Fig. 1). Because the level of pyruvate increases under anaerobic conditions and activates PTA toward acetylphosphate synthesis, anaerobic conditions favor the production of acetylphosphate. Therefore, PTA is a target of metabolic engineering to reduce acetate production and, subsequently, to increase succinate production in *E. coli* (25).

The Δ*pta* mutant of strain ES02 was designated ES03 (Table 1). The strains ES03/empty, ES03/PYC, and ES03/PCK were constructed and evaluated for fermentative products (Table 3). The acetate titer and yield were decreased in ES03/empty (0.5 g/liter corresponding to a 13.5% yield) compared to ES02/empty (1.8 g/liter corresponding to a 52.9% yield). However, the 2,3-butanediol titer in ES03/empty (1.4 g/liter corresponding to a 37.8% yield) was approximately 7-fold higher than that of ES02/empty (0.2 g/liter corresponding to a 5.9% yield).

The strains ES03/PYC and ES03/PCK produced 3.8 and 4.2 g/liter succinate from 8.9 and 7.4 g/liter consumed glucose, respectively, resulting in succinate yields of 42.7% and 56.8%, respectively (Table 3). Although inactivation of PTA was effective in decreasing acetate formation, it also decreased succinate production because of the increased 2,3-butanediol production in ES03/PYC and ES03/PCK.

**Effect of inactivating α-acetolactate decarboxylase on succinate production in ES03.** Our previous study showed that deletion of the *budA* gene encoding α-acetolactate decarboxylase eliminated both acetoin and 2,3-butanediol production (Fig. 1). The *budA* gene was therefore disrupted from strain ES03 to obtain ES04 (Table 1). The production of succinate in ES04/empty, ES04/PYC, and ES04/PCK is summarized in Table 3. Both the titer and yield of acetate and pyruvate in ES04/empty (pyruvate, 0.8 g/liter, corresponding to a 25.8% yield; acetate, 0.8 g/liter, corresponding to a 25.8% yield) were increased over 1.6 times compared to those in ES03/empty (pyruvate, 0.5 g/liter, corresponding to a 13.5% yield; acetate, 0.5g/liter, corresponding to a 13.5% yield).

Strains ES04/PYC and ES04/PCK produced 3.6 and 3.5 g/liter succinate from 6.2 and 5.8 g/liter consumed glucose, resulting in yields of 58.1% and 60.3%, respectively (Table 3). These succinate yield values were increased approximately 1.4- and 1.1-fold from those of ES03/PYC (42.7%) and ES03/PCK (56.8%), respectively. However, the succinate titers in ES04/PYC (3.6 g/liter) and ES04/PCK (3.5 g/liter) were slightly decreased from those in ES03/PYC (3.8 g/liter) and ES03/PCK (4.2 g/liter). Elimination of the 2,3-butanediol synthesis pathway slightly increased the succinate yield but decreased its titer because of increased pyruvate and acetate production in ES04/PYC and ES04/PCK.

A metabolic engineering approach based on the elimination of pyruvate node reactions involved in lactate, acetate, and 2,3-butanediol synthesis from the Δ*adhE*/PCK strain contributes to decreasing the total amount of by-products (Table 3). However, a decreased formation of by-products did not correlate with increased succinate production in strains overexpressing PYC or PCK (Fig. 2B).

**Coexpression of *C. glutamicum pyc* and *A. succinogenes pck* in ES02, ES03, and ES04.** To investigate the effect of coexpressing PCK and PYC on succinate production, the coexpression plasmid pSTV28-*pck-pyc* was introduced into strains ES02, ES03, and ES04. The succinate titer and yield in all strains was increased when either PYC or PCK was overexpressed but was greatest when they were coexpressed (Fig. 2B and C and Table 3). The highest production of succinate was observed in strain ES04/PCK+PYC. The succinate titer of this strain was 8.1 g/liter, which was approximately 2.3-fold higher than those of ES04/PCK (3.5 g/liter) and ES04/PYC (3.6 g/liter). The succinate yield in ES04/PCK+PYC reached 71.0% and was approximately 1.2-fold higher than those

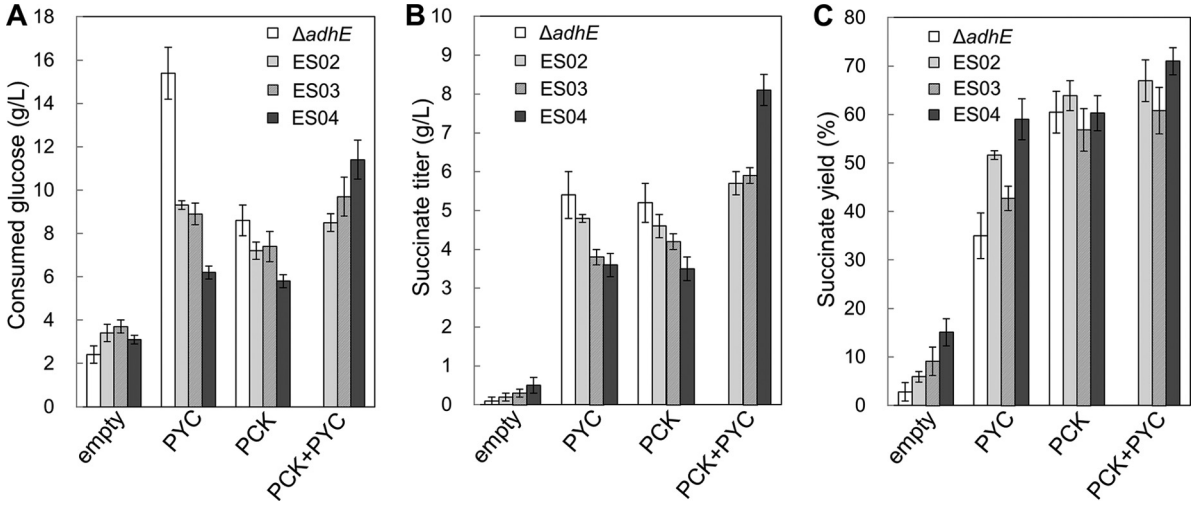


**FIG 2** Consumed glucose, succinate titer, and succinate yield of all strains using 1.5-ml microcentrifuge tube scale fermentation. (A) Consumed glucose (g/liter); (B) succinate titer (g/liter); (C) succinate yield (%). The values from the Δ*adhE*/empty, Δ*adhE*/PYC, and Δ*adhE*/PCK strains were excerpted from our previous work (18). Data are expressed as means ± standard deviations (SD).

of ES04/PYC (58.1%) and ES04/PCK (60.3%). The yields of pyruvate (2.6%) and acetate (5.2%) in ES04/PCK+PYC were slightly decreased from those in ES04/PYC (pyruvate, 3.2%; acetate, 6.5%) and ES04/PCK (pyruvate, 3.4%; acetate, 8.6%). Thus, this new coexpression system was more effective in increasing both the succinate titer and yield than when PCK and PYC were individually expressed.

**Succinate production in ES04/PCK+PYC under pH-controlled conditions.** To evaluate the impact on succinate production of lowering the culture pH from neutral to weakly acidic conditions, Strain ES04/PCK+PYC was cultured at pH 5.5, 5.7, or 7.0 in a 100-ml jar fermenter. The results are summarized in Fig. 3 and Table 4. At pH 5.7, the succinate titer and yield were 11.2 g/liter and 72.7%, respectively. These values and the profiles of end products were similar to those obtained in 1.5-ml microcentrifuge tubes (Table 3). Lowering the pH from 7.0 to 5.5 had negative effects on the succinate titer but positive effects on its yield (Fig. 3, Table 4). The succinate titer at pH 5.5 was 5.1 g/liter, which was only 22.6% of that at pH 7.0 (22.6 g/liter). In contrast, the succinate yield at pH 5.5 was 70.8%, 1.3 times higher than that at pH 7.0 (55.8%). At pH 7.0, the titers of pyruvate (5.1 g/liter) and malate (5.4 g/liter) were increased 10 and 27 times compared with those at pH 5.5, respectively. Thus, lowering the pH from 7.0 to a weakly acidic pH (pH 5.5 and 5.7) leads to a decreased succinate titer but increased yield.

## DISCUSSION

Two general metabolic engineering approaches have been used with various bacteria to increase carbon flux toward succinate synthesis: (i) elimination of pathways that compete for the carbon needed for succinate synthesis (19–21) and (ii) enhancement of carboxylation pathways from PEP and/or pyruvate to OAA (26–29). In the present study, both strategies were utilized, and the effects on succinate production in *E. aerogenes* were determined.

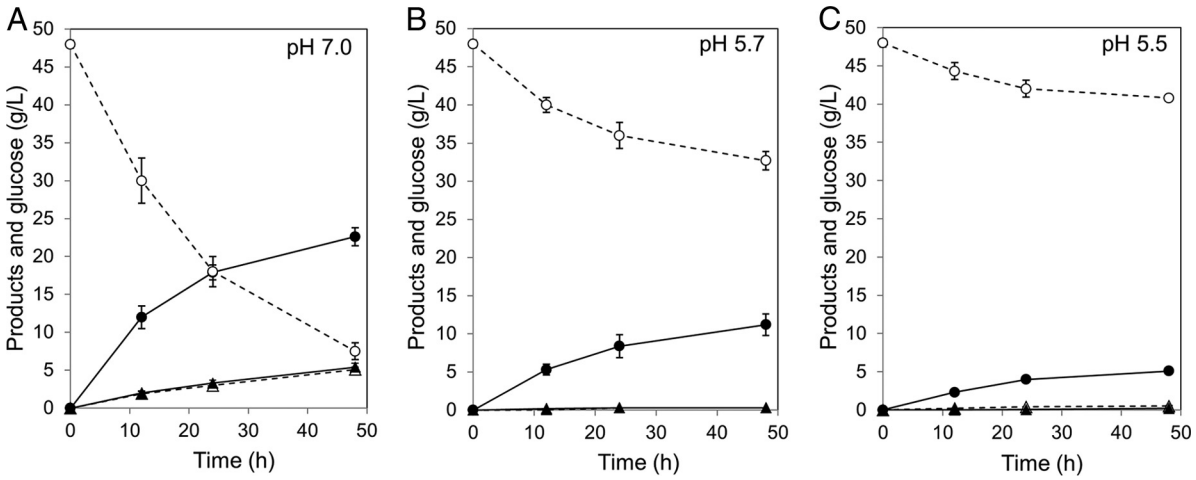


**FIG 3** Profiles of main products and glucose of strain ES04/PCK+PYC under pH-controlled conditions. The pH was maintained at 7.0 (A), 5.7 (B), and 5.5 (C). Symbols indicate glucose (open circles), succinate (closed circles), pyruvate (open triangles), and malate (closed triangles). Data from three independent experiments are shown. Error bars indicate ±SD.

**TABLE 4** Profiles of biomass, consumed glucose, end products, succinate yield, and volumetric productivity in strain ES04/PCK+PYC under pH-controlled batch culture conditions[a]

| Controlled pH[b] | Biomass (g [DCW]/liter)[c] | Consumed glucose (g/liter) | End product (g/liter) | | | | | | | | Succinate yield (% [g/g])[d] | Volumetric productivity (g/liter/h)[e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Pyruvate | Malate | Formate | Succinate | Lactate | Acetate | Ethanol | 2,3-Butanediol | | |
| 7.0 | 1.5 ± 0.1 | 40.5 ± 1.1 | 5.1 ± 0.3 | 5.4 ± 0.5 | 1.0 ± 0.1 | 22.6 ± 1.2 | 0.3 ± 0.1 | 1.1 ± 0.4 | 0.2 ± 0.1 | <0.1 | 55.8 ± 3.8 | 0.47 ± 0.02 |
| 5.7 | 1.3 ± 0.1 | 15.4 ± 1.2 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.1 ± 0.1 | 11.2 ± 1.4 | 0.1 ± 0.1 | 0.5 ± 0.1 | <0.1 | <0.1 | 72.7 ± 3.6 | 0.23 ± 0.03 |
| 5.5 | 1.2 ± 0.1 | 7.2 ± 0.4 | 0.5 ± 0.1 | 0.2 ± 0.1 | <0.1 | 5.1 ± 0.2 | 0.1 ± 0.1 | 0.4 ± 0.1 | <0.1 | <0.1 | 70.8 ± 0.5 | 0.11 ± 0.01 |

[a] Data are expressed as means ± SD from three independent experiments.
[b] pH was maintained at 7.0, 5.7, and 5.5 by 2 N NaOH.
[c] Initial biomass was adjusted to 1.2 g (DCW)/liter.
[d] Succinate yield is gram of product per gram of consumed glucose expressed as a percentage.
[e] Volumetric productivity indicates the succinate titer (g/liter/h).

As shown Fig. 2, an increased titer and yield of succinate were evident in strains coexpressing PCK and PYC. The extent of this increase depended upon which competing pathways were eliminated. These results strongly suggested that combination approaches, elimination of by-product pathways and this coexpression system, are necessary to maximally improve succinate production in *E. aerogenes*. Thus, strain ES04 (Δ*adhE* Δ*ldhA* Δ*pta* Δ*budA*)/PCK+PYC produced succinate with small amounts of by-products and over a 70% yield at pH 5.5 (Fig. 3, Table 4).

Earlier studies showed that elimination of pyruvate node reactions effectively increased succinate production in *E. coli* and *C. glutamicum* strains overexpressing PYC (20, 27, 35–37). In *E. aerogenes*, this approach increased the succinate yield but decreased its titer (Fig. 2B and C) (Table 3). The succinate yield and titer in strain ES04/PYC were 1.7 and 0.8 times, respectively, those of the "parental" Δ*adhE*/PYC strain. Removing the pyruvate node reactions increased the flux of the PYC-dependent reaction, thereby increasing the succinate yield. However, removing these reactions also promoted redox imbalance, resulting in decreased glucose consumption. For example, a decrease in glucose consumption was observed in the PYC-overexpressing strain when the lactate and 2,3-butanediol synthesis pathways, which are involved in NADH reoxidation, were inactivated (Fig. 2A). These results strongly suggested that succinate production in the strain overexpressing PYC was not limited by the supply of NADH.

Interestingly, eliminating the pyruvate node reactions in *E. aerogenes* strains overexpressing PCK decreased the succinate titer but did not influence the succinate yield, which was approximately 60% in the Δ*adhE*, ES02, ES03, and ES04 PCK-overexpressing strains (Fig. 2C). This phenomenon can be explained by the balance of PEP and pyruvate when glucose is imported via phosphotransferase (PTS), which is the predominant glucose uptake system in *E. aerogenes*. In the PTS system, the import of 1 mol of glucose yields 1 mol of pyruvate and PEP. A PCK-dependent reaction converts most of the PEP to OAA, while most of the pyruvate is converted to by-products in the PCK-overexpressing strains (Fig. 1). Overall, succinate yields in these strains were similar and approximately half of the maximum theoretical yield (112.3%).

In *E. aerogenes*, pyruvate can be presumably converted to PEP by PEP synthetase (PPS) when coupled with the conversion of ATP to AMP (Fig. 1). However, we speculate that this reaction is performed in an ATP-dependent manner and thus is largely inactive under energy-insufficient anaerobic conditions. Thus, the production of succinate in the strains overexpressing PCK is limited by the supply of PEP.

To overcome these factors limiting succinate production in strains overexpressing PYC or PCK, two carboxylation pathways from PEP and pyruvate to OAA were introduced (29). This approach was feasible because overexpressing PYC concurrently with PCK can recapture the pyruvate generated by glucose-PTS and direct it back to OAA. Subsequently, the increased OAA pool contributes to improve redox balance by effectively consumption of NADH via malate dehydrogenase (MDH) in succinate synthesis pathways (Fig. 1). We used *A. succinogenes* PCK and *C. glutamicum* PYC to create a new coexpression system in strains ES02, ES03, and ES04. This coexpression resulted in strains with a succinate yield and titer that were higher than those when either PYC or PCK was overexpressed individually (Fig. 2, Table 3). Overall, this expression system effectively functions to increase succinate production in these strains with eliminated pyruvate node reactions.

There are only a few previous reports on the impact of low pH (below pH 6.0) on succinate production in bacteria, even though this is considered economically favorable compared to neutral pH (7). As shown Fig. 3 and Table 4, at pH 5.5 strain ES04/PCK+PYC produced 5.1 g/liter succinate with a 70.8% yield. Interestingly, the succinate yield at pH 5.5 and 5.7 was approximately 1.3 times higher than that at pH 7.0 (55.8%). This is because the formation of excess pyruvate and malate was only observed at pH 7.0. These data suggested that downstream reactions from malate, such as fumarase (FUM), fumarate reductase (FRD), and the succinate exporter, limited succinate production under neutral pH conditions (Fig. 1). Although enhanced MDH activity leads to increased succinate production in *E. coli* (38), other downstream reactions, such as FRD, regulate succinate production in ES04/PCK+PYC (Fig. 1). FRD activity is induced via FNR (a DNA-binding transcriptional dual regulator) under anaerobic conditions in *E. coli* (39). Therefore, if anaerobic conditions are insufficient to induce FRD activity, an excess level of malate can be formed. The effect of decreasing malate production on succinate production by continuously expressing a high level of FRD in ES04/PCK+PYC remains to be determined.

The results from pH-controlled fermentation showed that a decreased pH decreased the succinate titer in strain ES04/PCK+PYC, similar to previous reports in *E. coli* (7, 40) (Fig. 3, Table 4). Maintaining a constant pH in the cell under acidic conditions requires more energy than under neutral conditions because of the demands imposed by acid resistance mechanisms, such as the proton efflux pump. This can decrease overall energy availability, thereby limiting succinate production under acidic and anaerobic conditions. To improve the production of succi-

nate under these conditions, genetic modifications based on an energy-conserving strategy may be effective. Previous studies indicated that replacing glucose uptake from PTS with glucose permease to increase the PEP supply resulted in an increase net yield of ATP because of the dominant PEP carboxylation by PCK with ATP generation in *E. coli* (41–44). In the near future, we will construct a further engineered strain based on the energy-conserving strategy and evaluate the impact of increase net ATP yield on succinate production under weakly acidic (pH <6.0) and anaerobic conditions. We believe that these studies will be useful to understand the relationship between the net yield of ATP and succinate production and to advance our understanding of bacterium-based succinate fermentation under acidic and anaerobic conditions.

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## REFERENCES

1. **Buschke N, Schäfer R, Becker J, Wittmann C.** 2013. Metabolic engineering of industrial platform microorganisms for biorefinery applications–optimization of substrate spectrum and process robustness by rational and evolutive strategies. Bioresour Technol **135:**544–554. http://dx.doi.org/10.1016/j.biortech.2012.11.047.
2. **Jang Y-S, Kim B, Shin JH, Choi YJ, Choi S, Song CW, Lee J, Park HG, Lee SY.** 2012. Bio-based production of C2-C6 platform chemicals. Biotechnol Bioeng **109:**2437–2459. http://dx.doi.org/10.1002/bit.24599.
3. **McKinlay JB, Vieille C, Zeikus JG.** 2007. Prospects for a bio-based succinate industry. Appl Microbiol Biotechnol **76:**727–740. http://dx.doi.org/10.1007/s00253-007-1057-y.
4. **Zeikus JG, Jain MK, Elankovan P.** 1999. Biotechnology of succinic acid production and markets for derived industrial products. Appl Microbiol Biotechnol **51:**545–552. http://dx.doi.org/10.1007/s002530051431.
5. **Cheng K-K, Zhao X-B, Zeng J, Wu R-C, Xu Y-Z, Liu D-H, Zhang J-A.** 2012. Downstream processing of biotechnological produced succinic acid. Appl Microbiol Biotechnol **95:**841–850. http://dx.doi.org/10.1007/s00253-012-4214-x.
6. **Li Q, Wang D, Wu Y, Li W, Zhang Y, Xing J, Su Z.** 2010. One step recovery of succinic acid from fermentation broths by crystallization. Sep Purif Technol **72:**294–300. http://dx.doi.org/10.1016/j.seppur.2010.02.021.
7. **Lu S, Eiteman MA, Altman E.** 2009. pH and base counterion affect succinate production in dual-phase *Escherichia coli* fermentations. J Ind Microbiol Biotechnol **36:**1101–1109. http://dx.doi.org/10.1007/s10295-009-0594-z.
8. **Samuelov NS, Lamed R, Lowe S, Zeikus JG.** 1991. Influence of CO(2)-HCO(3) levels and pH on growth, succinate production, and enzyme activities of *Anaerobiospirillum succiniciproducens*. Appl Environ Microbiol **57:**3013–3019.
9. **Van der Werf MJ, Guettler MV, Jain MK, Zeikus JG.** 1997. Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus sp*. 130Z Arch Microbiol **167:**332–342.
10. **Foster JW.** 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. Nat Rev Microbiol **2:**898–907. http://dx.doi.org/10.1038/nrmicro1021.
11. **Warnecke T, Gill RT.** 2005. Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications. Microb Cell Fact **4:**25. http://dx.doi.org/10.1186/1475-2859-4-25.
12. **Tsuji A, Okada S, Hols P, Satoh E.** 2013. Metabolic engineering of *Lactobacillus plantarum* for succinic acid production through activation of the reductive branch of the tricarboxylic acid cycle. Enzyme Microb Technol **53:**97–103. http://dx.doi.org/10.1016/j.enzmictec.2013.04.008.
13. **Zhang C, Lv F-X, Xing X-H.** 2011. Bioengineering of the *Enterobacter aerogenes* strain for biohydrogen production. Bioresour Technol **102:**8344–8349. http://dx.doi.org/10.1016/j.biortech.2011.06.018.
14. **Jung M-Y, Ng CY, Song H, Lee J, Oh M-K.** 2012. Deletion of lactate dehydrogenase in *Enterobacter aerogenes* to enhance 2,3-butanediol production. Appl Microbiol Biotechnol **95:**461–469. http://dx.doi.org/10.1007/s00253-012-3883-9.
15. **Nwachukwu R, Shahbazi A, Wang L, Ibrahim S, Worku M, Schimmel K.** 2012. Bioconversion of glycerol to ethanol by a mutant *Enterobacter aerogenes*. AMB Express **2:**20. http://dx.doi.org/10.1186/2191-0855-2-20.
16. **Converti A, Perego P.** 2002. Use of carbon and energy balances in the study of the anaerobic metabolism of *Enterobacter aerogenes* at variable starting glucose concentrations. Appl Microbiol Biotechnol **59:**303–309. http://dx.doi.org/10.1007/s00253-002-1009-5.
17. **Shin SH, Kim JY, Lee S, Um Y, Oh M-K, Kim Y-R, Lee J, Yang K-S.** 2012. Complete genome sequence of *Enterobacter aerogenes* KCTC 2190. J Bacteriol **194:**2373–2374. http://dx.doi.org/10.1128/JB.00028-12.
18. **Tajima Y, Kaida K, Hayakawa A, Fukui K, Nishio Y, Hashiguchi K, Fudou R, Matsui K, Usuda Y, Sode K.** 2014. Study of the role of anaerobic metabolism in succinate production by *Enterobacter aerogenes*. Appl Microbiol Biotechnol **98:**7803–7813. http://dx.doi.org/10.1007/s00253-014-5884-3.
19. **Jantama K, Zhang X, Moore JC, Shanmugam KT, Svoronos SA, Ingram LO.** 2008. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. Biotechnol Bioeng **101:**881–893. http://dx.doi.org/10.1002/bit.22005.
20. **Inui M, Murakami S, Okino S, Kawaguchi H, Vertès AA, Yukawa H.** 2004. Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J Mol Microbiol Biotechnol **7:**182–196. http://dx.doi.org/10.1159/000079827.
21. **Lee SJ, Song H, Lee SY.** 2006. Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. Appl Environ Microbiol **72:**1939–1948. http://dx.doi.org/10.1128/AEM.72.3.1939-1948.2006.
22. **Lee SY, Hong SH, Moon SY.** 2002. In silico metabolic pathway analysis and design: succinic acid production by metabolically engineered *Escherichia coli* as an example. Genome Inform **13:**214–223.
23. **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:**268–274. http://dx.doi.org/10.1016/j.mib.2006.03.001.
24. **Thakker C, Martínez I, San K-Y, Bennett GN.** 2012. Succinate production in *Escherichia coli*. Biotechnol J **7:**213–224. http://dx.doi.org/10.1002/biot.201100061.
25. **Sánchez AM, Bennett GN, San K-Y.** 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. http://dx.doi.org/10.1016/j.ymben.2005.03.001.
26. **Kim P, Laivenieks M, Vieille C, Zeikus JG.** 2004. Effect of overexpression of *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase on succinate production in *Escherichia coli*. Appl Environ Microbiol **70:**1238–1241. http://dx.doi.org/10.1128/AEM.70.2.1238-1241.2004.
27. **Okino S, Noburyu R, Suda M, Jojima T, Inui M, Yukawa H.** 2008. An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Appl Microbiol Biotechnol **81:**459–464. http://dx.doi.org/10.1007/s00253-008-1668-y.
28. **Wang J, Zhu J, Bennett GN, San K-Y.** 2011. Succinate production from different carbon sources under anaerobic conditions by metabolic engineered *Escherichia coli* strains. Metab Eng **13:**328–335. http://dx.doi.org/10.1016/j.ymben.2011.03.004.
29. **Lin H, San K-Y, Bennett GN.** 2005. Effect of *Sorghum vulgare* phosphoenolpyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*. Appl Microbiol Biotechnol **67:**515–523. http://dx.doi.org/10.1007/s00253-004-1789-x.
30. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K–12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
31. **Katashkina JI, Hara Y, Golubeva LI, Andreeva IG, Kuvaeva TM, Mashko SV.** 2009. Use of the lambda Red-recombineering method for genetic engineering of *Pantoea ananatis*. BMC Mol Biol **10:**34. http://dx.doi.org/10.1186/1471-2199-10-34.
32. **Peredelchuk MY, Bennett GN.** 1997. A method for construction of *E. coli* strains with multiple DNA insertions in the chromosome. Gene **187:**231–238. http://dx.doi.org/10.1016/S0378-1119(96)00760-3.
33. **Minaeva NI, Gak ER, Zimenkov DV, Skorokhodova AY, Biryukova**

**IV, Mashko SV.** 2008. Dual-in/out strategy for genes integration into bacterial chromosome: a novel approach to step-by-step construction of plasmid-less marker-less recombinant *E. coli* strains with predesigned genome structure. BMC Biotechnol **8:**63. http://dx.doi.org/10.1186/1472-6750-8-63.

34. **Fukui K, Koseki C, Yamamoto Y, Nakamura J, Sasahara A, Yuji R, Hashiguchi K, Usuda Y, Matsui K, Kojima H, Abe K.** 2011. Identification of succinate exporter in *Corynebacterium glutamicum* and its physiological roles under anaerobic conditions. J Biotechnol **154:**25–34. http://dx.doi.org/10.1016/j.jbiotec.2011.03.010.
35. **Gokarn RR, Evans JD, Walker JR, Martin SA, Eiteman MA, Altman E.** 2001. The physiological effects and metabolic alterations caused by the expression of *Rhizobium etli* pyruvate carboxylase in *Escherichia coli*. Appl Microbiol Biotechnol **56:**188–195. http://dx.doi.org/10.1007/s002530100661.
36. **Sánchez AM, Bennett GN, San K-Y.** 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. http://dx.doi.org/10.1021/bp049676e.
37. **Sánchez AM, Bennett GN, San K-Y.** 2006. Batch culture characterization and metabolic flux analysis of succinate-producing *Escherichia coli* strains. Metab Eng **8:**209–226. http://dx.doi.org/10.1016/j.ymben.2005.11.004.
38. **Liang L-Y, Liu R, Ma J, Chen K, Jiang M, Wei P.** 2011. Increased production of succinic acid in *Escherichia coli* by overexpression of malate dehydrogenase. Biotechnol Lett **33:**2439–2444. http://dx.doi.org/10.1007/s10529-011-0707-4.
39. **Tseng CP, Albrecht J, Gunsalus RP.** 1996. Effect of microaerophilic cell growth conditions on expression of the aerobic (cyoABCDE and cydAB) and anaerobic (narGHJI, frdABCD, and dmsABC) respiratory pathway genes in *Escherichia coli*. J Bacteriol **178:**1094–1098.
40. **Martínez I, Lee A, Bennett GN, San K-Y.** 2011. Culture conditions' impact on succinate production by a high succinate producing *Escherichia coli* strain. Biotechnol Prog **27:**1225–1231. http://dx.doi.org/10.1002/btpr.641.
41. **Zhang X, Jantama K, Moore JC, Jarboe LR, Shanmugam KT, Ingram LO.** 2009. Metabolic evolution of energy-conserving pathways for succinate production in *Escherichia coli*. Proc Natl Acad Sci U S A **106:**20180–20185. http://dx.doi.org/10.1073/pnas.0905396106.
42. **Singh A, Cher Soh K, Hatzimanikatis V, Gill RT.** 2011. Manipulating redox and ATP balancing for improved production of succinate in *E. coli*. Metab Eng **13:**76–81. http://dx.doi.org/10.1016/j.ymben.2010.10.006.
43. **Tang J, Zhu X, Lu J, Liu P, Xu H, Tan Z, Zhang X.** 2013. Recruiting alternative glucose utilization pathways for improving succinate production. Appl Microbiol Biotechnol **97:**2513–2520. http://dx.doi.org/10.1007/s00253-012-4344-1.
44. **Zhang X, Jantama K, Shanmugam KT, Ingram LO.** 2009. Reengineering *Escherichia coli* for succinate production in mineral salts medium. Appl Environ Microbiol **75:**7807–7813. http://dx.doi.org/10.1128/AEM.01758-09.
45. **Tajima Y, Keita F, Hashiguchi K.** August 2012. Method for producing an organic acid. US patent 8,247,201 B2.