Genetic Reconstruction of the Aerobic Central Metabolism in *Escherichia coli* for the Absolute Aerobic Production of Succinate

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Abstract: Most reported efforts to enhance production of the industrially valuable specialty chemical succinate have been done under anaerobic conditions, where *E. coli* undergoes mixed-acid fermentation. These efforts have often been hampered by the limitations of NADH availability, poor cell growth, and slow production. An aerobic succinate production system was strategically designed that allows *E. coli* to produce and accumulate succinate efficiently and substantially as a product under absolute aerobic conditions. Mutations in the tricarboxylic acid cycle (*sdhAB, icd, iclR*) and acetate pathways (*poxB, ackA-pta*) of *E. coli* were created to construct the glyoxylate cycle for aerobic succinate production. Experiments in flask studies showed that 14.28 mM of succinate could be produced aerobically with a yield of 0.344 mole/mole using 55 mM glucose. In aerobic batch reactor studies, succinate production rate was faster, reaching 0.5 mole/mole in 24 h with a concentration of 22.12 mM; further cultivation showed that succinate production reached 43 mM with a yield of 0.7. There was also substantial pyruvate and TCA cycle C_6 intermediate accumulation in the mutant. The results suggest that more metabolic engineering improvements can be made to this system to make aerobic succinate production more efficient. Nevertheless, this aerobic succinate production system provides the first platform for enhancing succinate production aerobically in *E. coli* based on the creation of a new aerobic central metabolic network.

Keywords: glyoxylate; pyruvate; succinate; metabolic pathway; gene inactivation; metabolic engineering; aerobic fermentation

INTRODUCTION

The valuable specialty chemical succinate and its derivatives have extensive industrial applications. It can be used as an additive and flavoring agent in foods, a supplement for pharmaceuticals, a surfactant, a detergent extender, a foaming agent, and an ion chelator (Zeikus et al., 1999). Currently, succinate is produced through petrochemical processes that can be expensive and can lead to pollution problems. Much effort has shifted toward making biocatalysts a viable and improved alternative for the production of succinate. The success of microbial fermentation coupled with the use of renewable carbohydrates would significantly improve the economics of the succinate market (Schilling, 1995).

Various strains such as *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, and *Escherichia coli* have been intensively studied for their potential as biocatalysts in succinate fermentation. The obligate anaerobe *A. succiniciproducens* has shown high potential for industrial-scale succinate production because of its high conversion yield and productivity when fermented with whey (Lee et al., 2000). However, *A. succiniciproducens* is not practical for commercial fermentation because it is unstable due to its tendency to degenerate, and requires environments absolutely free of oxygen for cultivation (Nghiem et al., 1999). *E. coli* has also been extensively genetically engineered through the use of recombinant DNA technology in recent years to show promising potential for succinate fermentation. *E. coli* naturally produces succinate as a minor fermentation product under anaerobic conditions (Clark, 1989). Under aerobic conditions, succinate is not produced as a by-product in *E. coli* and acetate is the main by-product. Numerous metabolic engineering strategies to enhance succinate production in *E. coli* have met with success. By amplifying enzymatic steps involved in the succinate pathway under anaerobic conditions, higher succinate production could be achieved. An example of this was shown when phosphoenolpyruvate carboxylase (*pepC*) from *E. coli* was overexpressed (Millard et al., 1996). Conversion of fumarate to succinate was improved by overexpressing native fumarate reductase (*frd*) in *E. coli* (Goldberg et al.,

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1983; Wang et al., 1998). Certain enzymes are not indigenous in *E. coli*, but can potentially help increase succinate production. By introducing pyruvate carboxylase (*pyc*) from *Rhizobium etli* into *E. coli*, succinate production was enhanced (Gokarn et al., 1998, 2000, 2001). Other metabolic engineering strategies also include inactivating competing pathways of succinate. When malic enzyme was overexpressed in the presence of inactivated pyruvate formate lyase (*pfl*) and lactate dehydrogenase (*ldh*), succinate became the major fermentation product (Stols and Donnelly, 1997; Hong and Lee, 2001). In this *pfl* and *ldh* mutant, there is a large pyruvate accumulation. Overexpression of malic enzyme in this mutant increased succinate production driven by the high pyruvate pool toward the direction of malate formation, which was subsequently converted to succinate. An inactive glucose phosphotransferase system (*ptsG*) in the same mutant strain ($pfl^-$and ldh^-) had also been shown to yield higher succinate production in *E. coli* (Chatterjee et al., 2001).

The various genetic improvements described above for succinate production have all been done under anaerobic conditions utilizing the mixed-acid fermentation pathways of *E. coli*. Unfortunately, anaerobic fermentation has inherent disadvantages that are difficult to surmount. Anaerobic conditions often cause poor cell growth and slow carbon throughput, and therefore low production rates. Succinate formation in mixed-acid fermentation is also hampered by the limitations of NADH availability, since 2 moles of NADH are required for every mole of succinate to be formed. Strategies to overcome the anaerobic barrier have included generating enough biomass under aerobic conditions, then switching to anaerobic conditions for succinate production. This was shown to be effective using a "dual-phase" fermentation system, in which initial aerobic growth phase was started, then followed by an anaerobic production phase (Vemuri et al., 2002a,b). Absolute aerobic production of succinate in *E. coli* until now has not been metabolically engineered to show feasibility. *Saccharomyces cerevisiae* has been shown to increase succinate production aerobically when succinate dehydrogenase (*sdh*) is disrupted to utilize the oxidative pathway of the TCA cycle for production (Arikawa et al., 1999). The capability to produce succinate under aerobic conditions would mean an active oxidative phosphorylation for generating energy with O_2 present as the electron acceptor. This would lead to higher biomass generation, faster carbon throughput, and product formation. In this study, we seek to develop an aerobic succinate production platform in *E. coli*, thus creating a new metabolic network for improving succinate production under aerobic conditions.

To create a novel succinate production system that functions under absolute aerobic conditions, the aerobic central metabolism of *E. coli* is genetically reconstructed. Pathway modeling and simulation were performed on the glycolysis, TCA cycle, and glyoxylate bypass to create a design for the network that would allow succinate to be produced as a by-product aerobically with a substantial achievable yield. Based on the design, various mutations were created. The two major acetate pathways (pyruvate oxidase (*poxB*), and acetate kinase-phosphotransacetylase (*ackA-pta*)) were inactivated to channel more carbon flux toward the TCA cycle (Fig. 1). In the TCA cycle, *sdhAB* and *icd* were inactivated to redirect the fluxes toward succinate (Fig. 1). The glyoxylate operon *aceBAK* repressor (*iclR*) was inactivated in order to activate the glyoxylate bypass in the TCA cycle as a detour for succinate production. Studies have shown that disruption of *iclR* dramatically induces expression of the *aceBAK* operon when grown on glucose (Gui et al., 1996). This is not the case when grown on acetate, since growth on acetate yields induction of *aceBAK* anyway (Gui et al., 1996). There are also other transcriptional regulators of the *aceBAK* operon, which include FadR (Maloy and Nunn, 1995), FruR (Chin et al., 1989), and the ArcAB system (Iuchi and Lin, 1988; Iuchi et al., 1989). The glyoxylate bypass, consisting of two enzymatic steps, is essential for growth on the two-carbon acetate or fatty acids because it prevents carbon loss as CO_2 in the TCA cycle (Kornberg, 1966). The first step is isocitrate lyase (*aceA*), which converts isocitrate to succinate and glyoxyate. Then, in the secondary step, malate synthase (*aceB*) condenses the glyoxylate with acetyl-CoA to form malate. The malate is then converted back to oxaloacetate (OAA). These steps bypass the two oxidative steps of the TCA cycle in which CO_2 is evolved. With these genetic modifications, the glyoxylate cycle is created which produces succinate as a by-product. This will form the basis of the platform for aerobic succinate production. Through this aerobic system, succinate is produced through pathways that do not require

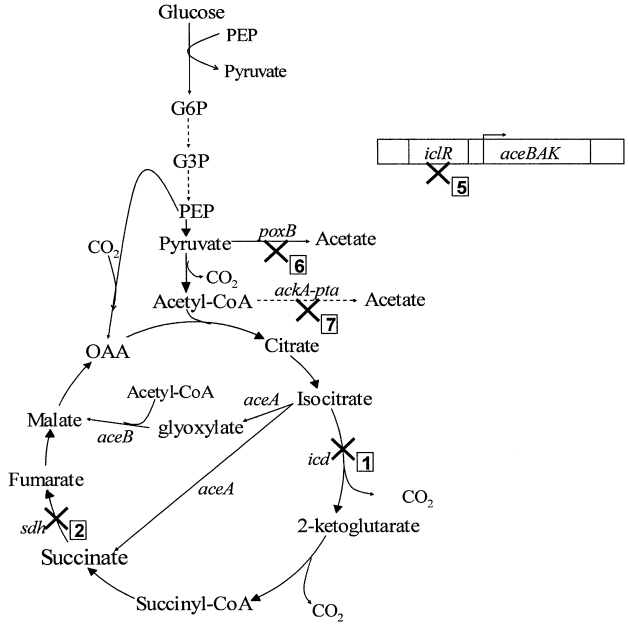

Figure 1. Genetic engineering of the glycolysis and TCA cycle in the development of an aerobic succinate production system. 1 is *icd* knockout, 2 is *sdh* knockout, 5 is *iclR* knockout, 6 is *poxB* knockout, and 7 is *ackA-pta* knockout.

NADH for formation. Cell growth and product formation would also be more efficient than anaerobic conditions. More important, the platform demonstrates the feasibility of producing succinate aerobically as a major product in *E. coli*.

MATERIALS AND METHODS

Strains

Mutations were created in the laboratory wildtype GJT001, a spontaneous *cadR* mutant of MC4100 (Tolentino et al., 1992). A library of mutants was created in the end throughout the process of constructing the pentamutant. A list of the mutant strains that were studied is shown in Table I. Knockouts were created in succinate dehydrogenase (*sdhAB*), pyruvate oxidase (*poxB*), acetate kinase-phosphotransacetylase (*ackA-pta*), isocitrate dehydrogenase (*icd*), and the *aceBAK* operon repressor (*iclR*). Strains HL2k, HL26k, HL267k, HL2671k, and HL26715k were used to characterize the effect of each sequential addition of a mutation to the previously studied host strain, GJT001. The final mutant, GJT001(Δ*sdhAB,* Δ*poxB,* Δ*(ackA-pta),* Δ*icd,* Δ*iclR*::Km), is the pentamutant that sets the basis for aerobic succinate production. Other pentamutant strains were also constructed using different orders of introduction of the mutations to confirm the phenotype of GJT001 (Δ*sdhAB,* Δ*poxB,* Δ*(ackA-pta),* Δ*icd,* Δ*iclR*::Km) in addition to genomic PCR verification. The Km resistant marker was left purposely on all the strains used in experiments for preventing contamination during cultivation with the use of kanamycin.

Mutant Construction

Mutations were created using the one-step inactivation method of Datsenko and Wanner (2000). This method first requires the construction of the single mutations using the phage λ Red recombinase. P1 phage transduction was then used to combine various mutations into one strain. Each mutation had to be added to the strain one at a time before the introduction of the next mutation because the kanamycin cassette had to be removed at each stage to enable selection of the next mutation. PCR products of the kanamycin cassette gene flanked by FRT (FLP recognition target) sites and homologous sequences to the gene of interest were made using pKD4 (Datsenko and Wanner, 2000) as the template. These PCR products were then transformed into the cells by electroporation (Bio-Rad, Hercules, CA, Gene Pulser) for insertional inactivation of the gene of interest. These transformed cells carry the plasmid pKD46 (Datsenko and Wanner, 2000) that expresses the λ Red system (γ, β, *exo*) for recombination of the PCR product into the chromosome. Once the kanamycin cassette is inserted it can be removed using the helper plasmid, pCP20 (Datsenko and Wanner, 2000), which expresses FLP. The removal of the FRT-flanked kanamycin cassette leaves behind an 84 basepair insertion cassette. At each stage of mutation, experiments were performed to test the intermediate mutant for the effect on metabolite production. Throughout the process of constructing the aerobic succinate production system, a library of different mutants with varying types and numbers of mutations was created. All mutants were also verified with genomic PCR after construction to ensure that the gene of interest had been disrupted.

Flask Culture Medium and Conditions

The base medium used for studying the mutants is LB (10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, 10 g/L NaCl) with 2 g/L of $NaHCO_3$ and 55 mM of glucose. The medium used for inoculum preparation is also LB, except glucose was not added. The pH of the medium was adjusted to 7.5 with NaOH. $NaHCO_3$ was added to the culture medium because it helped yield better cell growth and succinate production due to its pH-buffering capacity and its ability to supply CO_2. The aerobic setup of the experiments utilized 250-mL flasks with foam stoppers. Each

Table I. Table of mutant strains constructed and studied.

Strain	Genotype	Reference
GJT001	Spontaneous *cadR* mutant of MC4100(ATC35695) Δ*lac(arg-lac)*U169*rps*L150*relA*1*pts*F Sm^R	Tolentino et al., 1992
HL2k	GJT001 (Δ*sdhAB*::Km^R)	This study
HL26k	GJT001 (Δ*sdhAB,* Δ*poxB*::Km^R)	This study
HL267k	GJT001 (Δ*sdhAB,* Δ*poxB,* Δ*(ackA-pta)*::Km^R)	This study
HL2671k	GJT001 (Δ*sdhAB,* Δ*poxB,* Δ*(ackA-pta),* Δ*icd*::Km^R)	This study
HL26715k	GJT001 (Δ*sdhAB,* Δ*poxB,* Δ*(ackA-pta),* Δ*icd,* Δ*iclR*::Km^R)	This study
HL27615k	GJT001 (Δ*sdhAB,* Δ*(ackA-pta),* Δ*poxB,* Δ*icd,* Δ*iclR*::Km^R)	This study
HL12675k	GJT001 (Δ*icd,* Δ*sdhAB,* Δ*poxB,* Δ*(ackA-pta),* Δ*iclR*::Km^R)	This study
HL51267k	GJT001 (Δ*iclR,* Δ*icd,* Δ*sdhAB,* Δ*poxB,* Δ*(ackA-pta)*::Km^R)	This study
HL51276k	GJT001 (Δ*iclR,* Δ*icd,* Δ*sdhAB,* Δ*(ackA-pta),* Δ*poxB*::Km^R)	This study
HL52167k	GJT001 (Δ*iclR,* Δ*sdhAB,* Δ*icd,* Δ*poxB,* Δ*(ackA-pta)*::Km^R)	This study
HL52176k	GJT001 (Δ*iclR,* Δ*sdhAB,* Δ*icd,* Δ*(ackA-pta),* Δ*poxB*::Km^R)	This study

flask contained 50 ml of the base medium and a 1% (v/v) inoculum was used, grown overnight from glycerol stocks kept at −85°C. Kanamycin was added to the flask at a concentration of 50 mg/L. Cultures were grown at 37°C and 250 rpm for 24 h.

Bioreactor Culture Medium and Conditions

Aerobic batch reactor studies were conducted on the pentamutant HL27615k. The medium used is LB with 2 g/L $NaHCO_3$ and 63 mM of glucose. The initial medium volume is 600 ml in a 1.0-L New Brunswick Scientific (Edison, NJ) Bioflo 110 fermenter. A 1% (v/v) inoculum was used from an overnight culture grown from a single colony for 12 h. The pH was measured using a glass electrode and controlled at 7.0 using 1.5 N HNO_3 and 2 N Na_2CO_3. The temperature was maintained at 37°C and the agitation speed was constant at 800 rpm. The inlet airflow used was 1.5 L/min. The dissolved oxygen was monitored using a polarographic oxygen electrode and was maintained above 80% saturation throughout the experiment.

Analytical Techniques

Optical density was measured at 600 nm with a spectrophotometer (Bausch & Lomb, Rochester, NY, Spectronic 1001); the culture was diluted to the linear range with 0.15 M NaCl. For analyzing the extracellular metabolites, 1 ml of culture was centrifuged and the supernatant was then filtered through a 0.45-μm syringe filter for HPLC analysis. The HPLC system (Shimadzu-10A Systems, Columbia, MD) used was equipped with a cation-exchange column (HPX-87H, BioRad, Hercules, CA), a UV detector (Shimadzu SPD-10A), and a differential refractive index (RI) detector (Waters 2410, Milford, MA). A 0.6 mL/min mobile phase using 2.5 mM H_2SO_4 solution was applied to the column. The column was operated at 55°C. Standards were prepared for glucose, succinate, acetate, and pyruvate for both the RI detector and UV detector, and calibration curves were created. Glucose, succinate, and acetate were measured by the RI detector and pyruvate was measured by the UV detector at 210 nm.

RESULTS AND DISCUSSION

Design and Construction of the Aerobic Succinate Production System

Succinate is naturally formed as a product under anaerobic conditions where *E. coli* undergoes mixed-acid fermentation. Under aerobic conditions, the production of succinate as a product is not naturally possible. In aerobic metabolism, succinate is only an intermediate of the TCA cycle. It is formed by succinyl-CoA synthetase, and then subsequently converted to fumarate by succinate dehydrogenase. Through the oxidation reaction by succinate dehydrogenase (SDH), succinate also provides electrons to the electron transport chain for oxidative phosphorylation. Because of this recycling process, succinate is never detected in aerobic cultures of *E. coli*. Acetate is the only major by-product of *E. coli* under aerobic conditions. To enable production of succinate as a by-product under aerobic conditions, pathways in the central metabolism had to be redesigned by genetic manipulation. Pathways involved in the glycolysis, TCA cycle, and the glyoxylate bypass were engineered to create a platform for aerobic succinate production. First, to make succinate accumulation possible under aerobic conditions, succinate dehydrogenase (*sdh*) in the TCA cycle was inactivated (Fig. 1). Once *sdh* was inactivated, succinate started accumulating during aerobic growth. Next, to increase the carbon flux toward the TCA cycle for succinate production, two competing acetate pathways were inactivated (pyruvate oxidase (*poxB*), and acetate kinase-phosphotransacetylase (*ackA-pta*)) (Fig. 1). Once these two pathways were inactivated, acetate production decreased substantially, and more carbon flux was driven toward the TCA cycle.

The design of the aerobic production system involved redesigning the TCA cycle. To conserve the two carbons lost to CO_2 by the oxidative arm of the TCA cycle, the glyoxylate bypass was engineered as a route for succinate production. With this bypass, the two carbons were recovered as glyoxylate, which was then recycled back to malate to replenish oxaloacetate (OAA). This was made possible by first inactivating isocitrate dehydrogenase (*icd*) (Fig. 1). Once *icd* was inactivated, succinate could no longer be produced by the oxidative arm of the TCA cycle. Next, the glyoxylate bypass was activated by inactivating the *aceBAK* operon repressor (*iclR*) (Fig. 1). This caused the isocitrate lyase (*aceA*) and malate synthase (*aceB*) to be expressed constitutively. Upon these modifications, succinate could now be produced entirely through the glyoxylate bypass under absolute aerobic conditions.

The five mutations above were implemented, which together created a pentamutant of *E. coli*. Essentially, the TCA cycle is now the glyoxylate cycle, where succinate is being produced aerobically as a major by-product (Fig. 2). This production system provides a new platform for further improving succinate production in *E. coli* by utilizing the redesigned aerobic central metabolic pathways. The system has a maximum theoretical yield of producing 1 mole of succinate for every mole of glucose consumed.

Effect of Mutations on Aerobic Metabolite Production and Cell Growth

Experiments were conducted on each mutant strain throughout the construction of the pentamutant strain. The results of the pentamutant strain and its precursor mutant strains are based on the strain HL26715k (Table I). Each number in Figure 1 designates the specific gene that was inactivated, so each number in the strain name corresponds to a particular mutation. A total of five mutant strains and the

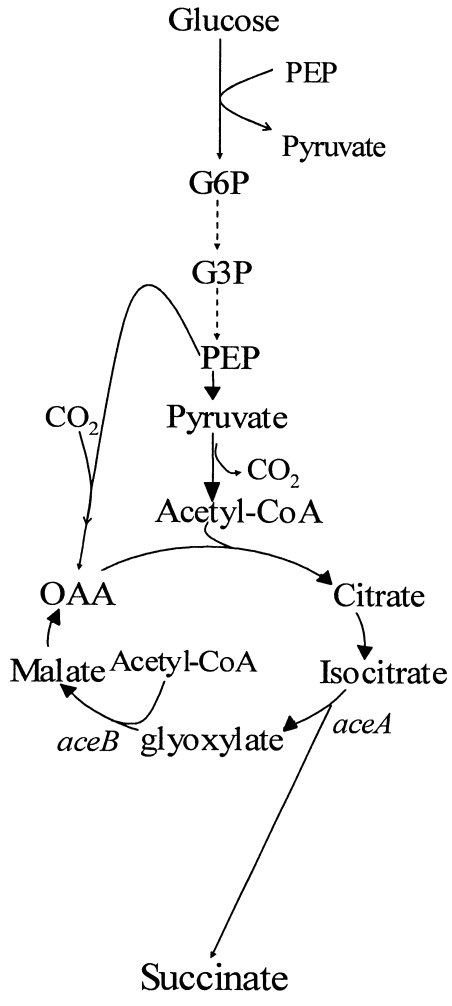

Figure 2. The glyoxylate cycle platform for aerobic succinate production.

parental strain (GJT001) were grown aerobically in flasks for 24 h at 37°C with 10 g/L glucose. Figure 3 shows the results of succinate production and yield from the culture of each mutant strain and the parental strain. In the mutant strain HL2k with only *sdh* inactivated, succinate accumulated during culture. Succinate accumulation was not possible in the wildtype (GJT001), as shown by zero succinate production (Fig. 3a). Inactivation of the two acetate pathways, *poxB* and *ackA-pta*, further increased succinate production and yield as shown by mutant strain HL267k. Next, as dictated by the design strategy, *icd* was inactivated creating mutant strain HL2671k. When *icd* was inactivated, succinate production decreased, as expected, since the cell probably could no longer use the oxidative arm of the TCA cycle to produce succinate. The amount of succinate produced by HL2671k could be due to the glyoxylate bypass being partially active. The molar succinate yield of HL2671k increased significantly, but this was accompanied by a much lower glucose consumption (Fig. 4a). Finally, when the glyoxylate bypass was activated by inactivating *iclR*, succinate production increased substantially to 14.28 mM with a molar yield of 0.344. This is over a 5-fold increase in succinate production compared to HL2671k. The result is shown by the pentamutant strain HL26715k. At this point, a highly functioning glyoxylate cycle is created. This provides a detour to relieve the carbon flux from the TCA cycle bottleneck created in mutant strain HL2671k. Activating the glyoxylate bypass reconstituted the cycling and replenishment of OAA. As a result, HL26715k showed much higher glucose consumption than the previous three strains containing mutations due to a faster and more efficient carbon throughput (Fig. 4a). The cell growth of HL26715k was also healthy again, and was similar to that of the wildtype parental strain GJT001 (Fig. 5a).

Glucose consumption decreased throughout the first four mutant strains (Fig. 4a). Once HL26715k was created, glucose consumption was much higher due to an active glyoxylate cycle, but still not as high as the wildtype parental strain. This was also the same trend observed for the OD, where HL26715k generated an OD similar to the parental strain (Fig. 5a). The results of the OD yield study, Figure 5b, also show that HL26715k has a healthy metabolism because of its glyoxylate cycle. OD yield relates to the amount of biomass generated per mole of glucose consumed. The OD yield of the parental strain is lower

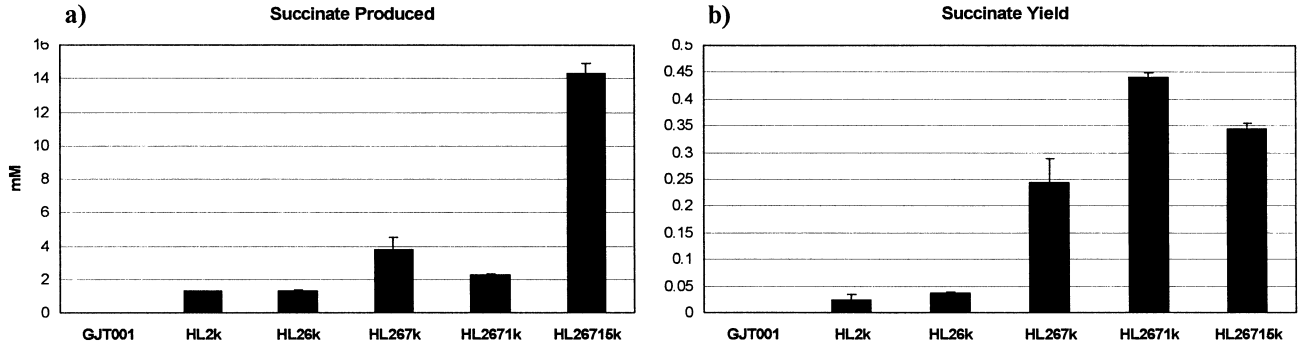

Figure 3. Succinate production in modified strains. **a:** Succinate production at each incremental step of incorporating mutations into the parental strain. The strain containing the five mutations combined represents the aerobic succinate production system. Each number designates a specific knockout in the pathways as shown by Figure 1. **b:** Succinate yield as a result of each incremental addition of mutation to the parental strain. Yield is mole of succinate produced per mole of glucose consumed. Mean and standard deviation were calculated based on duplicate experiments.

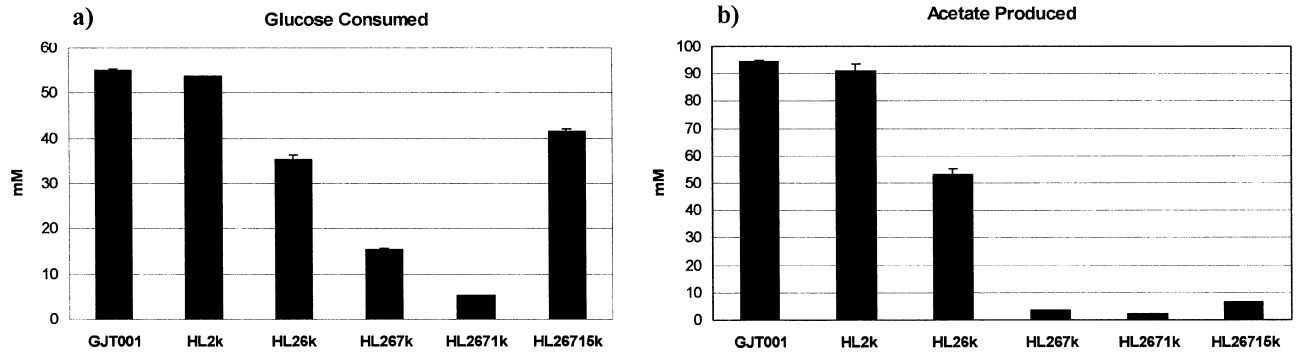

Figure 4. Glucose and acetate metabolism in modified strains. **a:** Glucose consumed by the parental strain and the five mutant strains after 24 h of culture. **b:** Acetate produced by the parental strain and the five mutant strains after 24 h of culture. Mean and standard deviation were calculated based on duplicate experiments.

than the OD yield of the five mutant strains (Fig. 5b). For the parental strain, this indicates that much of the consumed carbon source is being metabolized to end products rather than biomass. This would be due to the high carbon throughput of the central metabolism in the parental strain. In the mutant strain, the OD yield found in these cultures started to rise with each additional mutation up to the strain HL2671k. The OD yield for HL2671k was the highest among all the mutant strains. This is likely due to the inactivation of its two major acetate pathways and disruption of its entire TCA cycle. Because of these perturbations, the downstream part of HL2671k's central metabolism is curtailed. This probably resulted in slow carbon throughput, therefore lower glucose uptake and more carbon flux driven toward biomass. When the *iclR* was inactivated in HL2671k creating the pentamutant strain, HL26715k, the OD yield decreased significantly by 66% (Fig. 5b). The OD yield of HL26715k is also now closer to the OD yield of the wildtype parental strain. This result shows that activating the glyoxylate bypass reconstituted the cycling effect of the TCA cycle. In HL26715k, restoration of the downstream metabolism again allowed faster carbon throughput, higher glucose consumption, and less biomass generation per mole of glucose consumed.

Acetate production decreased significantly upon inactivation of *poxB* and *ackA-pta* (Fig. 4b). This was shown to direct more carbon flux downstream to succinate production. The intermediate, pyruvate, was observed to be accumulating significantly in strains HL26k, HL267k, HL2671k, and HL26715k (Fig. 6a). Inactivation of the two acetate pathways and disruption of the TCA cycle by *icd* knockout apparently caused a bottleneck at the pyruvate junction (Fig. 1). This is why there is substantial pyruvate accumulation in mutant strains HL267k and HL2671k based on the amount of glucose they consumed. The pyruvate yield is 2.398 (mole pyruvate/mole glucose) for HL267k and 2.274 (mole pyruvate/mole glucose) for HL2671k (Fig. 6b). When HL26715k was created by activating the glyoxylate bypass, the pyruvate yield decreased significantly to 0.338 (mole pyruvate/mole glucose) indicating a substantial relief of pyruvate accumulation by the glyoxylate cycle. The maximum theoretical pyruvate yield is 2.0 (mole pyruvate/mole glucose) generated from glycolysis. The high pyruvate yield above 2.0 exhibited by strains HL267k and HL2671k can be due to the effect of nutrients originating from the complex medium, which can be converted to pyruvate. In mutant strains with *icd* inactivated (HL2671k and HL26715k), there was also an accumulation of TCA

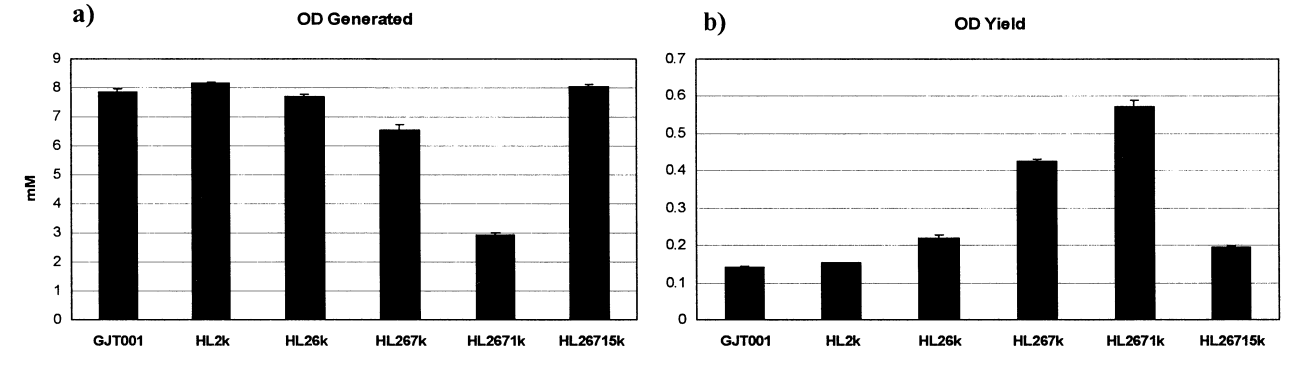

Figure 5. Growth of modified strains in standard media. **a:** Growth of the parental strain and the five mutant strains after 24 h. OD measured at 600 nm. **b:** OD yield after 24 h of culture. Yield is OD generated per mole of glucose consumed. Mean and standard deviation were calculated based on duplicate experiments.

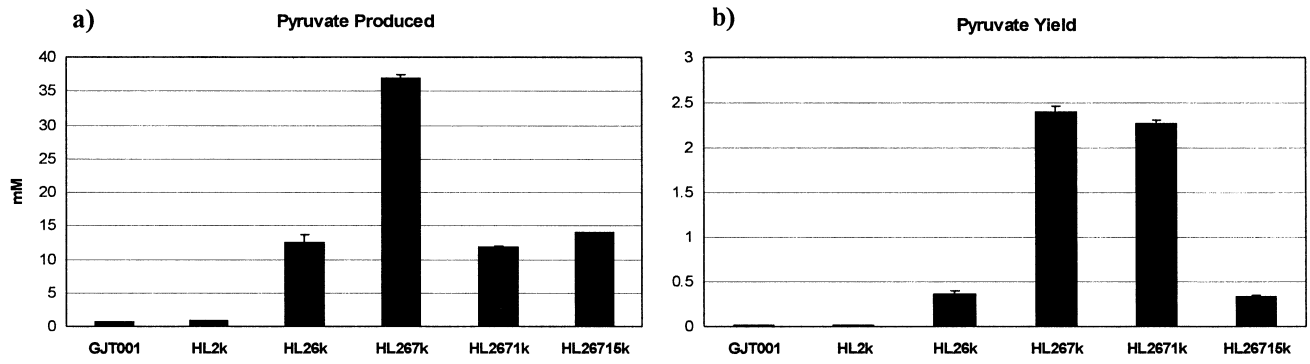

Figure 6. Pyruvate metabolism in modified strains. **a:** Accumulation of pyruvate by the parental strain and the five mutant strains after 24 h of culture. **b:** Pyruvate yield after 24 h of culture. Yield is mole of pyruvate produced per mole of glucose consumed. Mean and standard deviation were calculated based on duplicate experiments.

cycle C_6 intermediates such as citrate and isocitrate (data not shown). Others have shown similar effects when *icd* was inactivated in *E. coli* (Aoshima et al., 2003). This accumulation is less, though, in HL26715k than in HL2671k due to the glyoxylate cycle. Nevertheless, the presence of pyruvate and TCA cycle C_6 intermediate accumulation in the pentamutant strain HL26715k shows that there are significant improvements that can be made to this strain to further increase the succinate yield. This aerobic succinate production system will serve as a novel platform for future metabolic engineering improvements on succinate production in *E. coli*.

Verification of Pentamutant Construction

Throughout the process of constructing the pentamutant strain, a library of hosts with individual and multiple mutations was created by multistage phage transduction. To confirm that pentamutant strain's phenotype was correctly a result of the five mutations described in Figure 1 and not caused by artifacts from multiple phage transductions, seven different pentamutant strains were constructed with varying orders of input of the five mutations. Experiments in flask cultures were performed on these seven pentamutant strains to compare their metabolite production. The succinate production and yield of the seven pentamutant strains were very similar and consistent (Table II). These results show that the effect of the five mutations is indeed real and not affected by randomness of phage during transduction. This test further confirms the phenotype of the pentamutant strains.

Aerobic Batch Reactor Characterization of the Pentamutant

The pentamutant strain was further characterized under controlled conditions in an aerobic batch reactor using strain HL27615k. This would also demonstrate the possibility of using this aerobic succinate production system in an industrial setting. In the bioreactor, 63 mM of glucose was added and a 1% inoculum from an overnight culture grown from a single colony was used. Temperature and pH were maintained at 37°C and 7.0, respectively. The DO was maintained above 80% saturation.

The results show that at 24 h succinate production is 22 mM, with a molar yield of 0.5 (Fig. 7). This is better than the results from flask studies at 24 h, which were 14 mM of succinate with a yield of 0.34. Using a bioreactor generates higher productivity due to a more controlled environment. Cells reached maximum OD of 9.12 after 12 h, with a specific growth rate of $\sim 0.45\ h^{-1}$. At 24 h, pyruvate accumulation reached a maximum of 48 mM and glucose consumed was 44 mM. After 24 h, the cells started consuming the excreted pyruvate along with the remaining glucose. All the glucose was consumed by about 49 h, at which point the pyruvate was still being consumed. By 83 h, the pyruvate was not completely consumed, but succinate production reached 43 mM with a yield of 0.7. There was also accumulation of TCA cycle C_6 intermediates, which had not been consumed by the cells (data not shown). The results of the batch reactor study show that the pentamutant strain HL27615k has the potential to produce a large quantity of succinate under absolute aerobic conditions, and that there is potential to achieve the maximum suc-

Table II. Analysis of seven differently constructed pentamutant strains.

Pentamutant	Succinate produced (mM)	Succinate yield
HL12675k	14.33 ± 0.14	0.336 ± 0.024
HL26715k	16.07 ± 1.12	0.406 ± 0.039
HL27615k	16.49 ± 0.05	0.383 ± 0.005
HL51267k	16.29 ± 0.29	0.377 ± 0.010
HL51276k	15.64 ± 1.51	0.370 ± 0.001
HL52167k	16.40 ± 0.33	0.373 ± 0.003
HL52176k	13.32 ± 1.97	0.304 ± 0.028

Construction of seven different pentamutant strains that vary in order of input of each mutation. The resulting succinate produced and succinate yield are similar among all seven strains. Yield is calculated as mole of succinate produced per mole of glucose consumed. The strains were grown using 250-mL flasks with foam stoppers for 24 h in LB+55 mM of glucose. Mean and standard deviation were calculated based on triplicate experiments.

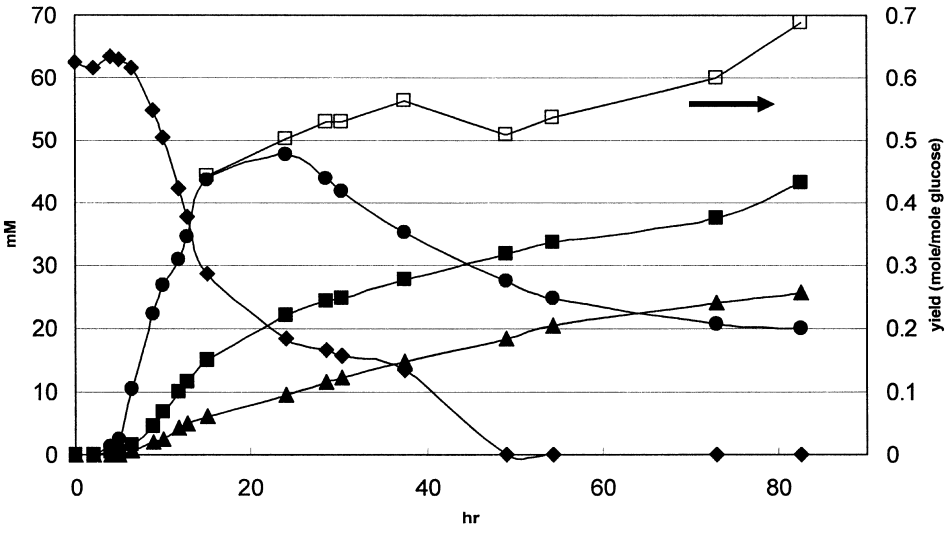

Figure 7. Aerobic batch reactor study on the pentamutant HL27615k. Solid diamond (♦) is glucose consumed. Solid square (■) is succinate produced. Solid triangle (▲) is acetate produced. Solid circle (●) is pyruvate produced. Open square (□) is the succinate yield (mole succinate produced per mole glucose consumed).

cinate theoretical yield of 1.0. Future improvements to this platform would be to increase the production rate and reduce the accumulation of intermediates. Various genetic modifications are under way to further enhance carbon throughput to succinate.

CONCLUSIONS

Although metabolic engineering of *E. coli* to enhance succinate production under anaerobic conditions has generated many promising improvements, they are still hampered by many inherent anaerobic constraints. This makes application on an industrial scale difficult due to low biomass generation and poor physiological state of the cell. An aerobic succinate production system is being developed to provide an alternative to anaerobic fermentation. Aerobic conditions provide many advantages that favor implementation on an industrial scale due to higher biomass generation, faster carbon throughput, and product formation.

The aerobic succinate production system is based on five mutations, Δ*sdhAB*, Δ*icd*, Δ*poxB*, Δ(*ackA-pta*), and Δ*iclR*. With these mutations in *E. coli*, a pentamutant strain is created with a new central metabolic network, i.e., the glyoxylate cycle. Through the glyoxylate cycle, succinate can be produced as a major by-product under aerobic conditions. Experiments have shown that the pentamutant strain HL26715k could produce substantial succinate under absolute aerobic conditions. HL26715k shown by its glucose consumption and cell growth is healthy and robust due to its glyoxylate cycle. The substantial accumulation of pyruvate and TCA cycle C_6 intermediates hinders achieving the maximum succinate theoretical yield of 1.0. These accumulations also presumably slowed down the production rate of succinate. There are significant improvements that can still be made to the system. Nevertheless, the current system provides the first platform for enhancing succinate production in *E. coli* under absolute aerobic conditions. Numerous metabolic engineering strategies can be further applied to this redesigned metabolic network for achieving the maximum succinate yield aerobically.

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