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Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions

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We examined succinic acid production in *Escherichia coli* AFP111 using dual-phase fermentations, which comprise an initial aerobic growth phase followed by an anaerobic production phase. AFP111 has mutations in the *pfl*, *ldh*A, and *pts*G genes, and we additionally transformed this strain with the *pyc* gene (AFP111/pTrc99A-*pyc*) to provide metabolic flexibility at the pyruvate node. Aerobic fermentations with these two strains were completed to catalog physiological states during aerobic growth that might influence succinate generation in the anaerobic phase. Activities of six key enzymes were also determined for these aerobic fermentations. From these results, six transition times based on physiological states were selected for studying dual-phase fermentations. The final succinate yield and productivity depend greatly on the physiological state of the cells at the time of transition. Using the best transition time, fermentations achieved a final succinic acid concentration of 99.2 g/l with an overall yield of 110% and productivity of 1.3 g/l h.
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Keywords: dual-phase fermentations; succinic acid; transition time; pyruvate carboxylase; pyruvate dehydrogenase; fumarate reductase

Introduction

The cellular physiology and the metabolic pathways used by microorganisms are vastly different under aerobic and anaerobic conditions [14,30,32,37,38]. Oxygen can be considered the most important regulatory signal in facultative anaerobes because of the broad spectrum of alternative pathways and enzymes, which are expressed under various levels of aerobic conditions [37,39]. *Escherichia coli* generates carbon dioxide as the major extracellular carbon product of aerobic growth, while the organism performs a mixed acid fermentation under anaerobic growth, accumulating formate, lactate, acetate, ethanol and to a smaller extent succinate [6]. Several genetic manipulations have been studied as means to increase succinate yield including deletion of the *ldhA* gene encoding fermentative lactate dehydrogenase [23], overexpression of the *ppc* gene for phosphoenolpyruvate (PEP) carboxylase [25], overexpression of the gene for malic enzyme [18,33,34], overexpression of the *pyc* gene for pyruvate carboxylase [11,12], and deletion of both pyruvate formate lyase (*pfl*) and *ldhA* genes [4]. A derivative of the *pfl ldhA* strain was isolated by selecting a spontaneous mutant that could grow anaerobically on glucose as the sole carbon source [9]. This strain, AFP111, was shown to have a mutation in the *ptsG* gene [5], which encodes for an enzyme of the phosphotransferase system (PTS). Because of the *ptsG* mutation, AFP111 relies on glucokinase for glucose uptake [41]. When grown aerobically for biomass generation and then subjected to anaerobic conditions (a "dual-phase" fermentation), AFP111 attains succinate yields and productivities of 0.99 and 0.87 g/l h, respectively [26]. Such dual-phase fermentations have the advantage of uncoupling growth and product formation, and thus unique operational conditions may be applied to each phase. Additionally, enzymes that carry out the biotransformations in the second non-growth-production phase are largely expressed during the aerobic growth phase and remain active throughout the production phase. Dual-phase fermentations are therefore not limited by the expression of only a select set of anaerobically induced enzymes, as in the case of a conventional exclusively anaerobic fermentation for succinate production by *E. coli*.

Like most other prokaryotes, *E. coli* replenishes carbon in the tricarboxylic acid (TCA) cycle via the enzyme PEP carboxylase. Since the product of this enzyme, oxaloacetate, leads directly to succinate via malate and fumarate, this anaplerotic reaction is vital to the accumulation of succinate. We recently demonstrated that the enzyme isocitrate lyase also plays an important role in the production of succinate in AFP111 [41]. The two biochemical pathways involved in the formation of succinate in *E. coli* AFP111 are shown in Figure 1. In order to attain the maximal succinate mass yield from glucose of 112%, the molar ratio of the carbon flux from fumarate to succinate to the carbon flux from isocitrate to succinate must be 5.0. Insufficient carbon flux through the PEP-to-fumarate branch or elevated carbon flux through the glyoxylate shunt lowers the observed yield [41]. Achieving the optimal ratio of fluxes in the two pathways involved in *anaerobic* succinate accumulation requires a concomitant balance in the activities of the participating enzymes, which have been expressed during *aerobic* growth. The global regulation of aerobic and anaerobic pathways is further complicated by the presence of different isozymes [3,14,19,32,37]. Since the specific activities of key enzymes may change during the progress of the aerobic phase, the physiological state at the time of

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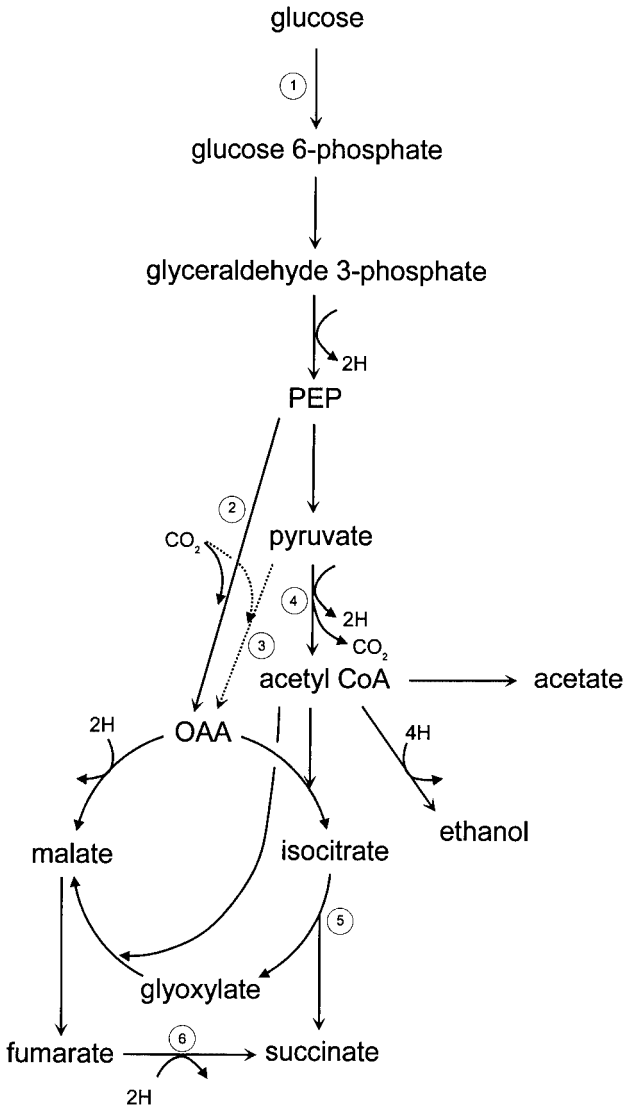

Figure 1 Metabolic pathways of *E. coli* AFP111 and AFP111/pTrc99A-*pyc* (dotted line) involved in the formation of succinate. Key enzymes assayed are: (1) glucokinase, (2) PEP carboxylase, (3) pyruvate carboxylase, (4) pyruvate dehydrogenase, (5) isocitrate lyase, (6) fumarate reductase.

the transition between the aerobic growth phase and the anaerobic production phase might prove important in the ultimate succinate yield and productivity.

Our objective was to study how the time of transition from aerobic to anaerobic phases affects succinate production in dual-phase fermentations by *E. coli* AFP111. Ideal milestones to mark such a transition would be readily measurable fermentation parameters that reflect the physiological state of the organism, such as respiratory quotient (RQ). We initially conducted exclusively aerobic fermentations merely to catalog "physiological milestones," which might be useful to mark a time of transition. Using these physiological signals as indicators of distinctive fermentation stages, we then performed dual-phase fermentations by making the transition to the anaerobic production phase at these milestones. We recently demonstrated that the presence of pyruvate carboxylase, an enzyme not found in wild-type *E. coli*, affords metabolic flexibility to AFP111 and allows this strain to achieve more readily the optimal molar ratio between the two succinate-generating pathways [41]. In the present study of dual-phase fermentations, we therefore also transformed AFP111 with the *pyc* gene and similarly cataloged fermentation stages. Thus, we determined how the time of transition and the presence of pyruvate carboxylase activity affect the formation of succinate.

Materials and methods

Strains and plasmids

E. coli AFP111 ($F^+\lambda^-$ *rpoS*396(Am) *rph*-1 Δ*pflAB*::Cam *ldh*A::Kan *ptsG*) was the only strain used in this study [5,9,26]. AFP111 was transformed with the *pyc* gene from *Rhizobium etli* using the pTrc99A-*pyc* plasmid (*R. etli pyc* Ap^R *trc*PO *lac*I^q Co1E1 ori) as described previously [13].

Fermentation media

All fermentations used a complex medium containing (g/l): glucose, 40; yeast extract, 10; tryptone, 20; $K_2HPO_4 \cdot 3H_2O$, 0.90; KH_2PO_4, 1.14; $(NH_4)_2SO_4$, 3.0; $MgSO_4 \cdot 7\ H_2O$, 0.30 and $CaCl_2 \cdot 2H_2O$, 0.25. The medium was supplemented with 1.0 mg/l biotin and 1.0 mg/l thiamine. For the *pyc*-containing strains the media also contained 100 mg/l ampicillin. Since significant pyruvate carboxylase activity exists without the addition of a chemical inducer [41], most studies were performed without inducer. For a final optimized fermentation, pyruvate carboxylase expression was induced to a greater level by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM.

Growth conditions

The 37°C fermentations had an initial volume of 1.5 l in 2.5-l Bioflow II fermenters (New Brunswick Scientific Instruments, New Brunswick, NJ). Inocula of 100 ml used the same medium as the fermenter and were grown in shake flasks for 6 h at 37°C. A series of exclusively aerobic fermentations (i.e., without a transition to anaerobic conditions) were first completed in order to catalog the changes in the physiological states of AFP111 and AFP111/pTrc99A-*pyc* during the aerobic growth phase. Constant agitation rates of 500 and 750 rpm were studied, corresponding to volumetric oxygen mass transfer coefficients ($k_L a$) of 52 and 69 h^{-1}, respectively, as calculated by the method of Taguchi and Humphery [36]. The air flow rate was maintained at 1.20 l/min by mass flow controllers (Unit Instruments, Orange, CA). The pH was controlled at 7.0 with 20% NaOH and 20% HCl. The dissolved oxygen concentration (DO) was monitored with an on-line probe (Mettler-Toledo Process Analytical Instruments, Wilmington, MA). The oxygen and CO_2 concentrations in the off-gas were measured by a gas analyzer (Ultramat 23, Siemens, Munich, Germany) and used to calculate the RQ. The activities of several key enzymes of the central metabolism were also measured at regular intervals during aerobic growth: glucokinase [28], PEP carboxylase [22], pyruvate carboxylase [29], pyruvate dehydrogenase [35], isocitrate lyase [8] and fumarate reductase [24]. Cell-free extracts were prepared by washing the cell pellet with an appropriate buffer and disrupting the suspended cells using a

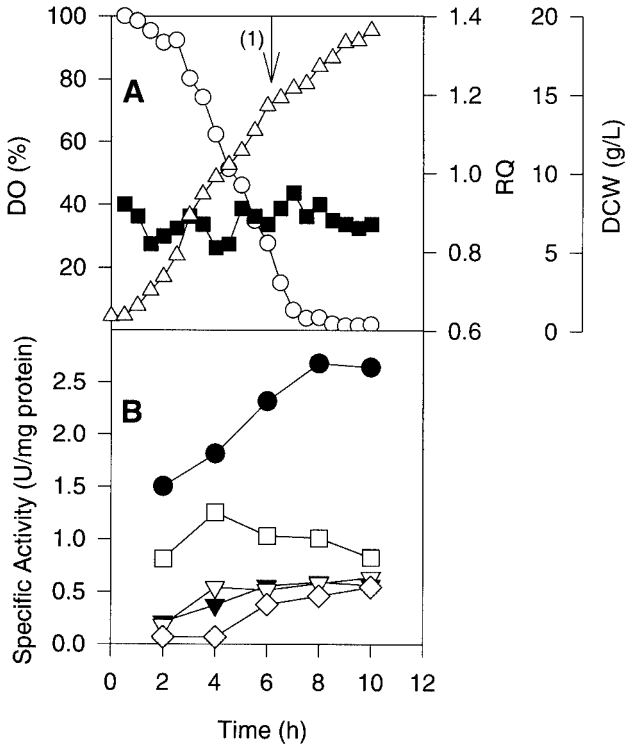

Figure 2 Aerobic fermentation of AFP111 at a medium value of k_La (52 h^{-1}). (A) Dry cell weight (DCW) (△), dissolved oxygen concentration (DO) (○) and respiratory quotient (RQ) (■). (B) The specific activities of the key enzymes: glucokinase (●), PEP carboxylase (□), pyruvate dehydrogenase (▼), isocitrate lyase (▽) and fumarate reductase (◇). Milestone (1) is shown.

French® pressure cell (ThermoSpectronic, Rochester, NY) at a pressure of 14,000 psi. Cell debris was removed by centrifugation (20,000×*g* for 15 min at 4°C), and the cell-free extract was used for measuring the enzyme activities. For all cases, one unit of enzyme activity is the quantity of enzyme that converts 1 μmol of substrate to product per minute at the optimum pH and temperature. Total protein in the cell-free extract was determined using bovine serum albumin as the standard [21]. Based on milestones observed in the course of these aerobic fermentations, several transition times were selected for further study.

Dual-phase fermentations were initiated as described for the aerobic fermentations. At each selected transition time, oxygen-free CO_2 was sparged at 0.2 l/min to replace air, and the agitation was reduced to 250 rpm. The pH was allowed to drift to 6.8, at which point it was controlled with 2.0 M Na_2CO_3. The glucose concentration was permitted to decrease to 3 g/l and then maintained at this level with an on-line analyzer (2700 Select, YSI, Yellow Springs, OH) by the controlled addition of a sterile 500 g/l glucose feed solution.

Analyses

Cell growth was monitored by measuring the optical density (OD) at 550 nm and correlating it with dry cell weight (DCW). Samples were centrifuged (10,000×*g* for 10 min at 25°C), and the supernatant analyzed for glucose and all products by high-pressure liquid chromatography (HPLC) as previously described [10].

Results

Physiological parameters during aerobic growth in the absence of pyruvate carboxylase

We first conducted exclusively aerobic fermentations using AFP111 in order to find distinguishable growth stages, and thereby define physiological "milestones" that could be used to transition to an anaerobic production phase. We compared these fermentations at k_La values of 52 h^{-1} (medium transfer rate) and 69 h^{-1} (high transfer rate). All fermentations were repeated three to six times, and consistent results were obtained with respect to the stages observed though a particular stage generally did not commence at one clock time. Representative fermentations are shown in the figures. Cell growth of AFP111 for medium transfer rate consistently exhibited three distinct stages (Figure 2A). Stage I corresponded to exponential growth (μ=0.7–0.8 h^{-1}), high DO and little acetate accumulation. Stage II corresponded to linear growth at 2 g/l h, decreasing DO and acetate accumulation at over 1 g/l h. Stage III corresponded to linear growth at 1.0–1.5 g/l h, oxygen limitation and less than 1 g/l h acetate accumulation. The specific enzyme activities of pyruvate dehydrogenase and isocitrate lyase increased substantially between stages I and II (Figure 2B). Also, fumarate reductase activity was very low until just prior to the onset of stage III. Because the intracellular levels of inhibitors and activators are not known, these *in vitro* enzyme activities indicate the level of active enzyme present but they do not necessarily indicate that carbon is flowing through a particular pathway.

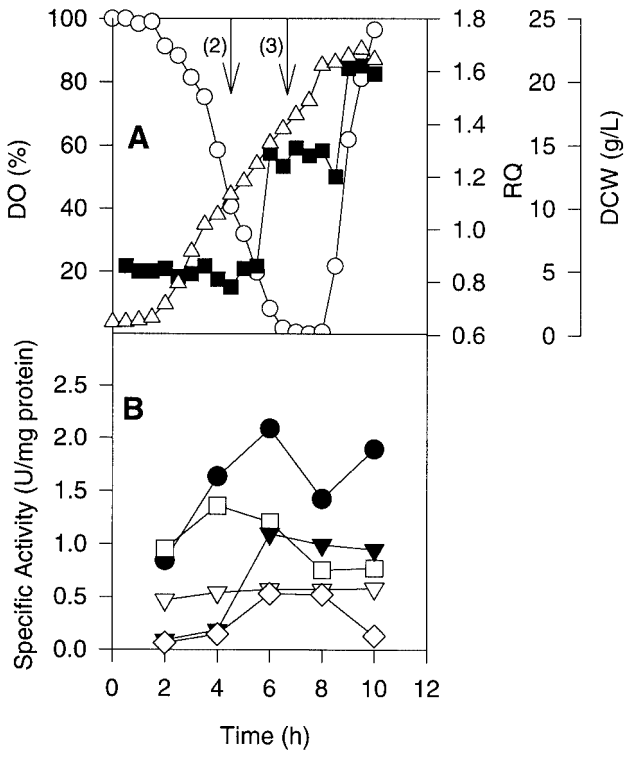

Figure 3 Aerobic fermentation of AFP111 at a high value of k_La (69 h^{-1}). (A) Dry cell weight (DCW) (△), dissolved oxygen concentration (DO) (○) and respiratory quotient (RQ) (■). (B) The specific activities of the key enzymes: glucokinase (●), PEP carboxylase (□), pyruvate dehydrogenase (▼), isocitrate lyase (▽) and fumarate reductase (◇). Milestones (2) and (3) are shown.

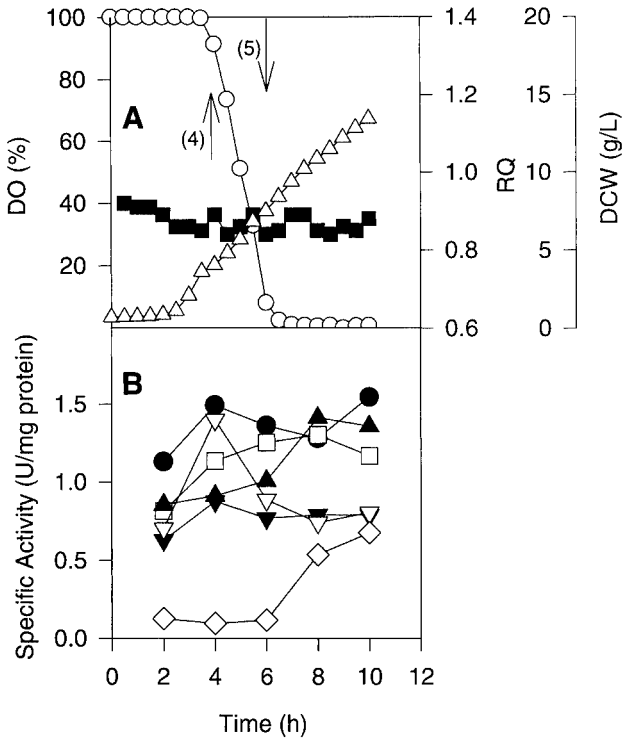

Figure 4 Aerobic fermentation of AFP111/pTrc99A-*pyc* at a medium value of k_La (52 h^{-1}). (A) Dry cell weight (DCW) (△), dissolved oxygen concentration (DO) (○) and respiratory quotient (RQ) (■). (B) The specific activities of the key enzymes: glucokinase (●), PEP carboxylase (□), pyruvate carboxylase (▲), pyruvate dehydrogenase (▼), isocitrate lyase (▽) and fumarate reductase (◇). Milestones (4) and (5) are shown.

For the fermentation of AFP111 at the high transfer rate (k_La=69 h^{-1}) the fermentations again followed three distinct stages (Figure 3A). Stage I was an exponential growth phase, with little acetate accumulation. Stage II was again marked by linear cell growth, but in contrast to results at medium transfer rate, acetate did not accumulate. Also, the RQ abruptly shifted from 0.8 to 0.9 to 1.2 to 1.3 when the DO reached about 10%, marking the start of the third stage. During stage III the cell growth rate remained at about 2.0 g/l h, the DO remained below 10%, the acetate concentration was negligible, and the RQ remained at 1.2–1.3. In general, the enzyme activities measured during the first 5–6 h were identical to those observed in the AFP111 fermentations at medium oxygen-transfer rate (Figure 3B). However, between stages II and III the activities of pyruvate dehydrogenase and fumarate reductase increased significantly. The activity of pyruvate dehydrogenase for these fermentations during the third stage (1.1 U/mg) was about twice that for the medium transfer rate fermentations, and over eight times greater than during stages I and II in the same fermentation. Also, PEP carboxylase activity decreased by over 30% from stage II to stage III. Because the pyruvate dehydrogenase complex generates carbon dioxide while PEP carboxylase consumes carbon dioxide, the increased RQ observed during phase III may be a consequence of a net increase in carbon dioxide generated by the change in activity of these two enzymes.

Based on these aerobic fermentations of AFP111, we identified three different milestones that could be used to mark a transition between growth and production phases. These times were selected because they were readily distinguishable and broadly represented the observed growth and enzyme activities. The first transition time studied (1) was at conditions of medium transfer rate as the fermentations entered stage III and the DO reached about 10–20% (indicated in Figure 2A). The second physiological time (2) was at conditions of high transfer rate with the fermentation in stage II (RQ still low), while the third time (3) was taken to be about 1.0 h after the initiation of stage III when RQ shifted (Figure 3A).

Physiological parameters during aerobic growth in the presence of pyruvate carboxylase

We completed aerobic fermentations of AFP111 with pyruvate carboxylase activity at the two different values of k_La (52 and 69 h^{-1}). For AFP111/pTrc99A-*pyc* at medium transfer rate, the DO consistently remained at 90–100% for about 5 h compared to the 2–3 h that had been observed for AFP111 at the same transfer rate (Figure 4A). However, because of the lower cell growth rate for AFP111/pTrc99A-*pyc*, in both cases the decrease in DO commenced when the cell concentration was about 5 g/l. We observed two distinct stages in these AFP111/pTrc99A-*pyc* fermentations. The first stage corresponded to high DO and exponential cell growth. The second stage commenced when the DO decreased substantially, and was marked by linear cell growth at about 1.5 g/l h. The specific activity of fumarate reductase increased after 6 h, but the other enzymatic activities did not appear to follow any trend (Figure 4B). Furthermore, throughout the fermentation the activity of glucokinase was substantially lower for AFP111/pTrc99A-*pyc* than we observed for AFP111, while the

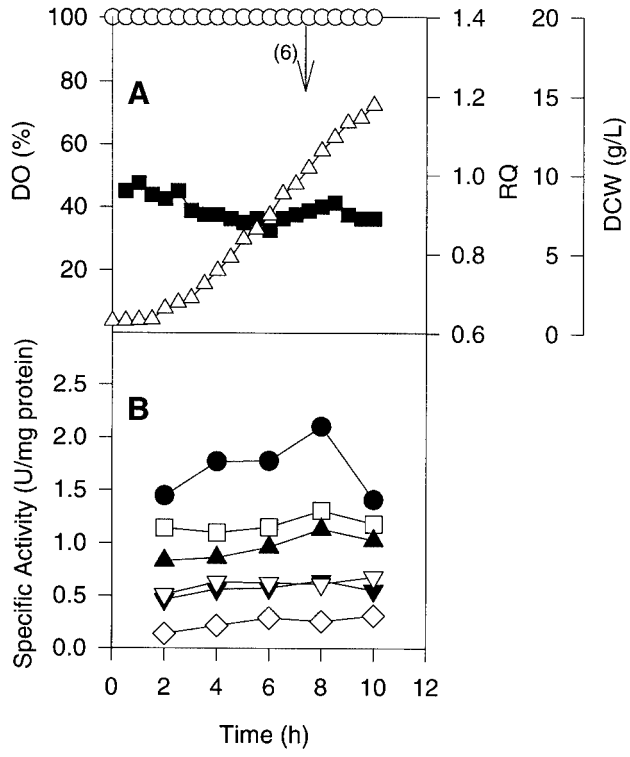

Figure 5 Aerobic fermentation of AFP111/pTrc99A-*pyc* at a high value of k_La (69 h^{-1}). (A) Dry cell weight (DCW) (△), dissolved oxygen concentration (DO) (○) and respiratory quotient (RQ) (■). (B) The specific activities of the key enzymes: glucokinase (●), PEP carboxylase (□), pyruvate carboxylase (▲), pyruvate dehydrogenase (▼), isocitrate lyase (▽) and fumarate reductase (◇). Milestone (6) is shown.

Table 1 Physiological milestones marking the transition between an aerobic growth phase and an anaerobic production phase in the fermentations of *E. coli* AFP111 and AFP111/pTrc99A-pyc (see Figures 2–5)

Milestone no.	Strain	k_La (h^{-1})	Physiological transition time
1	AFP111	52	Shift to a lower, linear cell-growth rate, DO about 20%, increased activity of fumarate reductase
2	AFP111	69	DO about 40–50%, RQ remains at 0.85
3	AFP111	69	DO less than 5%, RQ has shifted to 1.25, increased activity of fumarate reductase and pyruvate dehydrogenase
4	AFP111/pTrc99A-*pyc*	52	Linear cell-growth rate, DO has begun to decrease but is still about 90%
5	AFP111/pTrc99A-*pyc*	52	Linear cell growth rate, DO about 20%, increased activity of fumarate reductase
6	AFP111/pTrc99A-*pyc*	69	8.0 h

activities for all other enzymes were greater for AFP111/pTrc99A-*pyc* than we observed for AFP111.

Fermentations of AFP111/pTrc99A-*pyc* at high transfer rate were markedly different than those fermentations for AFP111 (Figure 5A). Specifically, the DO concentration remained at 100% and the RQ at 0.8–0.95 throughout the entire course of the fermentations, and cell growth proceeded at a constant rate. The enzyme activities (Figure 5B) also did not indicate any dramatic shift during the course of the fermentation. It is interesting to compare the DO for the aerobic fermentations at the time that the cell concentration had reached about 12 g/l. The DO at this cell density was consistently about 40% for AFP111 with a medium transfer rate, similar for AFP111 with a high transfer rate, essentially 0% for AFP111/pTrc99A-*pyc* with the medium transfer rate, but was invariably 100% for AFP111/pTrc99A-*pyc* at the high transfer rate.

Based on these results, we identified three different physiological milestones during AFP111/pTrc99A-*pyc* fermentations. The first transition time (4) was at conditions of medium transfer rate the DO began to decrease (Figure 4A). The second physiological time (5) was also at conditions of medium transfer rate with the fermentation strongly oxygen limited (DO less than 10%). Because there was no clear distinguishing physiological state during the fermentations of AFP111/pTrc99A-*pyc* at high transfer rate, the time of transition (6) was arbitrarily selected to be at 8.0 h, the only one of the six milestones based on clock time rather than a distinguishable physiological event. Table 1 summarizes the six milestones examined, three transition times for AFP111 and three transition times for AFP111/pTrc99A-*pyc*.

Dual-phase fermentations

We next studied dual-phase fermentations, which included a transition to an anaerobic production phase at each of the six milestones selected from exclusively aerobic fermentations. These fed-batch fermentations were routinely terminated after 48 h. AFP111 fermentations used milestones 1–3, while AFP111/pTrc99A-*pyc* fermentations used milestones 4–6 (Table 1). The results of these dual-phase fermentations are summarized in Table 2. The succinate yield was calculated as the mass of product formed in the anaerobic phase divided by the mass of glucose consumed in the anaerobic phase. The specific succinate productivity during the anaerobic phase was calculated on the basis of the cell concentration at the moment of transition. Generally, total cell mass in the fermenter (taking into account the dilution volume by the glucose feed) decreased by about 10% for AFP111 during the 40-h anaerobic production phase. In all cases for AFP111/pTrc99A-*pyc*, however, the total cell mass increased slightly (5–10%) during the course of the anaerobic phase. Fermentations using milestones 1, 4 and 5 resulted in significantly greater volumetric productivities than the other three fermentations. Thus, both AFP111 and AFP111/pTrc99A-*pyc* showed greater succinate productivity in an anaerobic phase when the preceding aerobic phase occurred at the medium oxygen-transfer rate than when aerobic growth occurred at the high oxygen-transfer rate. Since AFP111/pTrc99A-*pyc* grew more slowly than AFP111 under the conditions studied, the specific rate of succinate production was the greatest (118 mg/g h) for the fermentation with AFP111/pTrc99A-*pyc* and milestone 4. The yield of succinate was generally much greater for fermentations using AFP111/pTrc99A-*pyc* than AFP111.

Table 2 Comparison of fed-batch fermentations at the six milestones

Milestone	Strain	Q_P (g/l h)	q_P (mg/g h)	$Y_{S/G}$ (g/g)	S:A(g/g)
1	AFP111	1.21 ad	72 a	0.96 acd	10.5 ac
2	AFP111	0.51 b	35 b	0.45 b	6.7 ab
3	AFP111	0.84 c	47 be	0.89 a	7.6 b
4	AFP111/pTrc99A-*pyc*	1.29 a	118 c	1.14 c	8.0 b
5	AFP111/pTrc99A-*pyc*	1.11 d	89 d	1.13 c	7.1 b
6	AFP111/pTrc99A-*pyc*	0.78 c	54 ae	1.07 d	10.3 c

See Table 1 for details of each milestone. Q_P is the volumetric succinate productivity (g/l h) during the anaerobic phase; q_P is the specific succinate productivity (mg/g h) during the anaerobic phase; $Y_{S/G}$ is the mass yield of succinate based on glucose consumed during the anaerobic phase; S:A is the mass ratio of succinate to acetate present at the end of the fermentation. Parameters in a column followed by differing letters show statistically significant difference at the 90% confidence level.

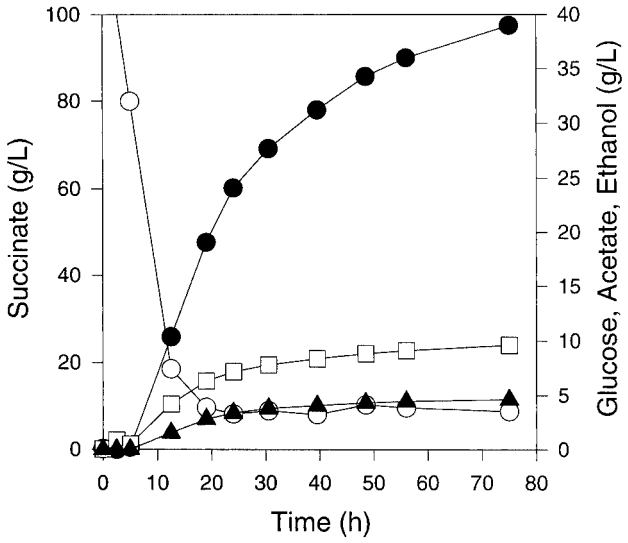

Figure 6 Fed-batch dual-phase fermentation of AFP111/pTrc99A-*pyc* at a medium value of $k_L a$ (52 h^{-1}) using milestone 4 as the time of transition. Glucose (○), succinate (●), acetate (□), ethanol (▲) concentrations are shown.

Dual-phase fermentations using the six milestones also generated other products. For AFP111, transitioning at milestone 1 resulted in pyruvate (0.6 g/l) and ethanol (2 g/l). In contrast, milestone 2 resulted in no pyruvate and less than 1 g/l ethanol, but fumarate to as much as 7 g/l. Milestone 3 resulted in similar pyruvate and ethanol (1 g/l). For AFP111/pTrc99A-*pyc*, no fumarate or pyruvate was observed in any case, and ethanol reached 2 g/l for milestones 4 and 6, but less than 1 g/l for milestone 5. Milestone 2 was the only transition time that consistently resulted in fumarate accumulation, a result that accounts for the particularly low succinate yield.

As milestone 4 appears to be the most promising for succinate production of the six studied, we conducted an extended fed-batch fermentation with AFP111/pTrc99A-*pyc* (Figure 6). The final succinate concentration was 97.5 g/l (99.2 g/l succinic acid). The volume of the fermentation increased from 1.5 to 2.5 l as a result of glucose feed and base addition. The succinate mass yield based on glucose consumed during the anaerobic phase alone was 117%. The overall succinate yield was 110%, and the overall volumetric succinate productivity was 1.3 g/l h. The final mass ratio of succinate to acetate was 10.2, and that of succinate to ethanol was 21. The cell mass concentration was 10.2 g/l at the transition between the growth and production phases. Based on this cell concentration, the specific succinate productivity for the anaerobic phase was 135 mg/g h. Accounting for the dilution volume, cells continued to grow throughout the anaerobic production phase, increasing in mass by 27%.

Discussion

We report here that physiological changes during aerobic growth of two engineered strains of *E. coli*, AFP111 and AFP111/pTrc99A-*pyc*, significantly affect succinate production in a subsequent production phase. Different aerobic operational conditions, such as oxygen-transfer rates ($k_L a$), would generally be expected to result in different levels of enzyme activity. Moreover, physiological states of an organism can change during the course of aerobic growth as the growth environment changes (for example, through oxygen limitation or product accumulation). These states often become evident in readily measurable parameters such as RQ, DO, component generation and utilization rates, and enzyme activities. The optimal transition time between the two fermentation phases appears to depend on the complex interplay of the activities of numerous enzymes in the two pathways central to succinate production.

For example, fumarate reductase activity changed during the course of the aerobic growth phases. Fumarate reductase is under the positive regulatory control of the FNR protein, which controls anaerobic metabolism in *E. coli* [3,32,37,39]. Under oxygen limitation, the FNR protein triggers the expression of fumarate reductase. In our studies with AFP111, increased fumarate reductase activity was observed at both oxygen-transfer rates as the fermentations became increasingly oxygen limited. The transition to a production phase prior to oxygen limitation and, thus, prior to an increased fumarate reductase activity (transition time 2) resulted in accumulation of fumarate at the expense of succinate. In our studies with AFP111/pTrc99A-*pyc*, fumarate reductase activity similarly increased with oxygen limitation for the medium transfer rate. However, for AFP111/pTrc99A-*pyc* with high transfer rate, we never observed a decrease in DO, and no increase in fumarate reductase activity was noted. In contrast with our results using AFP111, transition before oxygen limitation and significant fumarate reductase activity with AFP111/pTrc99A-*pyc* (milestones 4 and 6) did not result in fumarate accumulation. Indeed, milestone 4 resulted in the greatest succinate production of the six studied even though the transition was made prior to significant fumarate reductase activity observed in the exclusively aerobic fermentation. One might have expected increased pyruvate carboxylase activity to lead to an increase in fumarate accumulation at the expense of succinate. The presence of pyruvate carboxylase in this case seems to contribute to some other effect, such as a lower metabolic growth rate. Also, cells continued to grow slightly into the production phase with AFP111/pTrc99A-*pyc*, and thus enzymes likely could have been expressed in this second phase.

With exclusively aerobic fermentations of AFP111 at the high transfer rate, we consistently observed an abrupt shift in RQ with a simultaneous increase in the specific activity of pyruvate dehydrogenase. Also, AFP111 at the high transfer rate was never observed to accumulate acetate, while AFP111 at the medium transfer rate (and generally lower pyruvate dehydrogenase activity) accumulated significant acetate. It is widely believed that when the TCA cycle cannot keep pace with glycolysis, acetate accumulates, a phenomenon known as overflow metabolism [1,2,16,20,31]. Our observations indicate that high pyruvate dehydrogenase activity does not necessarily correlate with increased aerobic acetate production. With respect to succinate accumulation in a subsequent anaerobic phase, increased pyruvate dehydrogenase activity would cause carbon to flow into the glyoxylate shunt, a result that would diminish fumarate accumulation, but that ultimately would not provide maximal succinate yield. The cause of the relatively sudden expression of pyruvate dehydrogenase and its regulation are not known [7], although in our study activity of this enzyme increased, like fumarate reductase, with the onset of oxygen limitation. Under anaerobic conditions, the activity of pyruvate dehydrogenase is believed to be absent because of the low regeneration of NADH

[7,15], and all the carbon from pyruvate proceeds only through pyruvate formate lyase [40]. In the case of AFP111 and AFP111/pTrc99A-*pyc*, however, pyruvate is metabolized despite inactivation of the *pfl* gene encoding for pyruvate formate lyase [41]. Moreover, aerobically induced pyruvate dehydrogenase retains activity into a subsequent anaerobic phase. A similar report of anaerobic pyruvate metabolism in *E. coli* by pyruvate dehydrogenase for a low *in vivo* ratio of NADH/NAD [7], demonstrates that under anaerobic conditions, pyruvate metabolism in *pfl* mutants is possible in the presence of CO_2 and acetate. Of course, mutants in *pfl* require two-carbon intermediates for biosynthesis [40].

Another important enzyme is isocitrate lyase, which is necessary for carbon to flow to succinate via the glyoxylate shunt and is commonly associated with acetate metabolism [17]. This enzyme is not active under anaerobic conditions in *E. coli* AFP111 or AFP111/pTrc99A-*pyc* [41]. However, these two strains have significant isocitrate lyase activity under aerobic growth, and this activity is retained in the subsequent anaerobic production phase. For AFP111 growing aerobically at medium oxygen-transfer rate, acetate accumulated to over 7 g/l, while for the other conditions acetate did not accumulate. Considering that isocitrate lyase activity was similar for the two strains and two oxygen-transfer rates, it is not clear from our results why acetate would have consistently accumulated under one specific set of circumstances but not the other three.

Both AFP111 and AFP111/pTrc99A-*pyc* yielded the highest succinate productivities when the aerobic portion occurred at the medium oxygen-transfer rate. This result suggests that the physiological role of oxygen is central to establishing succinate productivity during the anaerobic phase. The presence of oxygen leads to the formation of certain harmful by-products such as peroxide, superoxide and hydroxyl radicals leading to oxidative stress [27]. In order to overcome the oxidative stress cells can produce antioxidants such as cysteine and glutathione, which would not be required under anaerobic conditions [27]. If such compounds are generated in the aerobic portion of a dual-phase fermentation, they may affect the subsequent anaerobic phase.

The presence of pyruvate carboxylase poses an extra burden for the cell and more energy for cell maintenance is needed in AFP111/pTrc99A-*pyc* than in AFP111. This additional burden would seem to account for the diminished cell-growth rate. Interestingly, the presence of pyruvate carboxylase at high oxygen-transfer rates prevented oxygen limitation from occurring during the entire growth phase. The presence of pyruvate carboxylase and its effect of slowing the growth rate may be the cause of decreased oxygen demand. However, at medium oxygen-transfer rates, the presence of pyruvate carboxylase appears to hasten the onset of oxygen limitation. Moreover, the RQ shifted from 0.8 to 1.2 only in one case (AFP111 with high oxygen-transfer rate) suggesting that path the process takes to oxygen limitation affects the state of the organism in the oxygen-limited stage. Additional studies with accurate measurement of specific oxygen uptake in the two strains under various oxygen limitations would seem necessary to reconcile these observations.

In summary, dual-phase fermentations permit the generation of high cell density in one phase, while generating product with high yield and productivity in a second phase. We have applied this type of fermentation to the production of succinic acid by *E. coli* and determined that the ideal time of transition between the growth and production phases for a desired product must be carefully selected based on physiological conditions at that moment.

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