Efficient Succinic Acid Production from Glucose through Overexpression of Pyruvate Carboxylase in an Escherichia coli Alcohol Dehydrogenase and Lactate Dehydrogenase Mutant

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An adhE, ldhA double mutant Escherichia coli strain, SBS110MG, has been constructed to produce succinic acid in the presence of heterologous pyruvate carboxylase (PYC). The strategic design aims at diverting maximum quantities of NADH for succinate synthesis by inactivation of NADH competing pathways to increase succinate yield and productivity. Additionally an operational PFL enzyme allows formation of acetyl-CoA for biosynthesis and formate as a potential source of reducing equivalents. Furthermore, PYC diverts pyruvate toward OAA to favor succinate generation. SBS110MG harboring plasmid pHL413, which encodes the heterologous pyruvate carboxylase from Lactococcus lactis, produced 15.6 g/L (132 mM) of succinate from 18.7 g/L (104 mM) of glucose after 24 h of culture in an atmosphere of CO₂ yielding 1.3 mol of succinate per mole of glucose. This molar yield exceeded the maximum theoretical yield of succinate that can be achieved from glucose (1 mol/mol) under anaerobic conditions in terms of NADH balance. The current work further explores the importance of the presence of formate as a source of reducing equivalents in SBS110MG(pHL413). Inactivation of the native formate dehydrogenase pathway (FDH) in this strain significantly reduced succinate yield, suggesting that reducing power was lost in the form of formate. Additionally we investigated the effect of ptsG inactivation in SBS110MG(pHL413) to evaluate the possibility of a further increase in succinate yield. Elimination of the ptsG system increased the succinate yield to 1.4 mol/mol at the expense of a reduction in glucose consumption of 33%. In the presence of PYC and an efficient conversion of glucose to products, the ptsG mutation is not indispensable since PEP converted to pyruvate as a result of glucose phosphorylation by the glucose specific PTS permease EIIICBα can be redvert toward OAA favoring succinate production.

Introduction

Succinic acid has drawn much interest because it has been used as a precursor of numerous chemicals including pharmaceuticals and biodegradable polymers (Hong and Lee, 2002). Succinic acid is a member of the C4-dicarboxylic acid family and is commercially manufactured by hydrogenation of maleic anhydride to succinic anhydride, followed by hydration to succinic acid. Recently major efforts have been made to produce succinic acid by microbial fermentation using a renewable feedstock (Lee et al., 2000; Lee et al., 2001; Hong and Lee, 2002). Nonrecombinant organisms have been isolated and studied, including anaerobic rumen bacteria such as Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes and Mannheimia succiniciproducens among others. However rumen organisms are characteristically unstable in fermentation processes (Nghiem et al., 1999). Other numerous attempts have been made to metabolically engineer the anaerobic central metabolic pathway of Escherichia coli to increase succinate yield and productivity. E. coli is extensively used in industry as a host for many products due to the ease of genetic manipulation coupled to its fast growth rate, standardized cultivation techniques and cheap media. It is for this reason and for the need to produce succinic acid economically at high concentrations and yields that E. coli has been considered as a potential candidate to produce this product of industrial interest.

Various efforts have been made to make succinate the major fermentation product in E. coli. Some genetic manipulations previously studied are deletion of the fermentative lactate dehydrogenase (LDH) pathway (Mat-Jan et al., 1989), deletion of both the LDH and pyruvate formate lyase (PFL) pathways (Bunch et al., 1997). and deletion of multiple pathways including PFL and LDH pathways with an additional ptsG mutation, which restored the ability of the strain to grow fermentatively on glucose and resulted in increased production of succinic acid (Donnelly et al., 1998; Chatterjee et al., 2001). Other studies include overexpression of phosphoenolpyruvate carboxylase (PEPC) (Millard et al., 1996), overexpression of the malic enzyme (Stols and Donnelly, 1997; Hong and Lee, 2000), overexpression of pyruvate carboxylase (PYC) (Gokarn et al., 1998; Gokarn et al.,
2000; Vemuri et al., 2002a), and overexpression of the heterologous Actinobacillus succinogenes phosphoenolpyruvate carboxykinase in a PEPC E. coli mutant (Kim et al., 2004). Besides these genetic manipulations, external means have been developed in order to increase succinate production such as utilizing a dual phase fermentation production mode which comprises an initial aerobic growth phase followed by an anaerobic production phase (Nghiem et al., 1999; Vemuri et al., 2002a; Vemuri et al., 2002b), and/or by changing the headspace conditions of the anaerobic fermentation using carbon dioxide, hydrogen or a mixture of both. It has been suggested that an external supply of H₂ might serve as a potential electron donor for the formation of succinic acid, a highly reduced fermentation product when compared to glucose (Vemuri et al., 2002a). In this paper we have used a dual phase fermentation production mode as described previously (Vemuri et al., 2002a) with slight modifications. The inoculum preparation consisted of growing the cells aerobically to generate sufficient biomass, followed by complete removal of the aerobic medium by centrifugation and resuspension of the cell mass in fresh anaerobic medium. Fully anaerobic conditions were established immediately with CO₂ sparged through the culture to displace air. All results presented in this paper are from the anaerobic production phase.

It is well-known that under anaerobic conditions and in the absence of exogenous electron acceptors, E. coli metabolizes glucose to a mixture of fermentative products consisting primarily of acetate, ethanol, lactate and formate with smaller quantities of succinate. NADH produced by the catabolism of glucose is regenerated to NAD⁺ through the reduction of intermediate metabolites derived from glucose in order to continue with glycolysis. The distribution of products varies according to the strain and growth conditions and is dictated by the way reducing equivalents generated in the form of NADH are consumed so that an appropriate redox balance is achieved by the cell (Clark, 1989; Chatterjee et al., 2001).

Under completely anaerobic conditions, the maximum theoretical yield (molar basis) of succinate from glucose is one based on the number of reducing equivalents provided by this substrate. One mole of glucose can provide only two moles of NADH, and two moles of NADH can only produce one mole of succinate; therefore, to surpass the maximum theoretical yield it is necessary to use part of the carbon coming from glucose to provide additional reducing power to the system.

In this study a genetically engineered E. coli strain has been created to achieve high succinate yield and productivity. The strategic design aims at diverting maximum quantities of NADH for succinate synthesis by striking a balance between cell physiology requirements and achieving higher product yields. As depicted in Figure 1, we deactivated two crucial enzymes in the central anaerobic pathway, LDH and alcohol dehydrogenase (ADH; AdhE), and overexpressed the heterologous PYC from Lactococcus lactis. The AdhE protein of E. coli is responsible for three different enzymatic activities. Two of these, ADH and coenzyme A-linked acetaldehyde dehydrogenase (ACDH), are involved in the conversion of acetyl-CoA to ethanol during fermentation (Gupta et

![Figure 1. Central anaerobic metabolic pathway of SBS110MG showing inactivation of LDH and ADH pathways and overexpression of the heterologous pyruvate carboxylase (PYC) from Lactococcus lactis.](https://example.com/fig1.png)
Antibiotic concentration. A volume of this culture was centrifuged, and the cells collected were resuspended in 18 mL of anaerobic medium to an initial OD600 of 4. The cells were transferred aseptically to 45-mL glass anaerobic tubes containing 0.5 g of MgCO3. The resuspended culture was sparged with sterile CO2 at 1 L/min STP for 8 s and rapidly capped with open top caps and PTFE/silicone rubber septa to ensure anaerobic conditions. A sample of the initial unincubated media was saved for analysis and samples were withdrawn with a syringe at 48, 96, and 168 h.

Larger Inoculum Experiments (20 OD600). Aerobic cultures were grown in a 2-L shake flask containing 400 mL of LB medium with appropriate antibiotic concentration. A volume of this culture was centrifuged, and the cells collected were resuspended in 10 mL of anaerobic medium to an initial OD600 of 20. The cells were transferred aseptically to 250 mL shake flasks containing 0.5 g of MgCO3. The resuspended culture was sparged with sterile CO2 at 1 L/min STP for 1 min and rapidly capped with rubber stoppers to ensure anaerobic conditions. For larger inoculum experiments, the use of shake flasks allowed a greater CO2/liquid ratio avoiding CO2 limitation conditions. A sample of the initial media was saved for analysis and samples were withdrawn with a syringe at 24 and 48 h.

Analytical Techniques. Cell density was measured at 600 nm in a spectrophotometer. Fermentation samples were centrifuged for 3 min at 13,000g in a microcentrifuge. The supernatant was filtered through a 0.45-μm syringe filter and stored chilled for HPLC analysis. The fermentation products as well as glucose were quantified using a Shimadzu HPLC system, equipped with a cation-exchange column (HPX-87H, BioRad Labs), a UV detector and a differential refractive index detector (Waters). A mobile phase of 2.5 mM H2SO4 solution at a 0.6 mL/min flow rate was used and the column was operated at 55°C.

Results

Effect of PYC Overexpression. The current study investigates the effect of expressing the heterologous PYC in a doubly mutated strain of E. coli, SBS110MG. Anaerobic tube experiments were performed under a complete atmosphere of CO2 using an initial OD600 of 4 with strain SBS110MG with and without plasmid pHL413 to assess the effect of overexpressing the pyc gene. Samples taken at different time intervals (48, 96, and 168 h) indicated that the expression of the pyc gene (plasmid pHL413) was necessary to increase the glucose uptake and to obtain high succinate yields. Fermentations with SBS110MG(pTrc99A) and SBS110MG(pHL413) were terminated after 168 h. At this point the control strain consumed only 11% of the initial glucose added (20 g/L) with low succinate yield and high acetate yield while SBS110MG(pHL413) consumed 100% of the initial glucose achieving a succinate yield of 1.3 mol/mol.

The effect of inoculum size on succinate production was also examined by using an inoculum of 20 OD600. Figure 2 shows the results of these experiments, including glucose consumed, the concentration of the metabolites produced and the product yields after 48h of culture.

A comparison of the results for SBS110MG (pTrc99A) and SBS110MG(pHL413) shows the effect of overexpressing pyc on the metabolic patterns of SBS110MG. The glucose consumption increased 4-fold; the succinate increased 25-fold from 5 mM to 132 mM from an initial glucose concentration of 104 mM. As expected, overexpression of pyc increased the succinate yield from 0.2

Table 1. List of Strains and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Phenotype</th>
<th>Source</th>
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<tr>
<td>MG1655</td>
<td>Wild type</td>
<td>ATCC 47076</td>
</tr>
<tr>
<td>SBS110MG</td>
<td>adhE, ldhA, Km8</td>
<td>This study</td>
</tr>
<tr>
<td>SBS220MG</td>
<td>adhE, ldhAmyG, Km8</td>
<td>This study</td>
</tr>
<tr>
<td>SBS880MGK</td>
<td>adhE, ldhAΔadhF, KmK</td>
<td>This study</td>
</tr>
<tr>
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<td>SBS770MGK</td>
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<td>Y. Zhao</td>
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<td>Plasmids</td>
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<td>pH413</td>
<td>Pyruvate carboxylase from Lactococcus lactis cloned in pTrc99A, ApR</td>
<td>Lin et al. 2004</td>
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Materials and Methods

Bacterial Strains and Plasmids. Table 1 describes the strains and plasmids used in this study. Single mutations were performed individually on MG1655 using the λ Red recombinase method of chromosomal disruption (Datsenko and Wanner, 2000). Additional mutations were introduced by P1-phage transduction with subsequent elimination of the kanamycin resistance gene. Single gene disruption sites were verified by PCR. Plasmid pH413 contains the pyc gene from Lactococcus lactis (Lin et al., 2004), which encodes the enzyme pyruvate carboxylase that converts pyruvate to oxaloacetate (OAA).

Cultivation Medium. Luria-Bertani (LB) broth medium supplemented with 66.7 mg/L each of ampicillin, carbenicillin, and oxacillin was used for all aerobic cultivations.

LB broth medium supplemented with 20 g/L of glucose and 1 g/L of NaHCO3 was used for all anaerobic cultivations and ampicillin was added at a concentration of 200 mg/L. Pyruvate carboxylase expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM.

Growth Conditions. A two-stage culture technique was used to examine the accumulation of succinic acid in the culture broth. The first stage comprises an initial aerobic growth phase followed by the second stage, the anaerobic production phase. Cells were grown aerobically in LB broth containing appropriate antibiotic concentration and incubated at 37 °C and 250 rpm for 17 h. Cells were harvested by centrifugation and the supernatant discarded. Then the cells were resuspended in fermentation medium at two different cell densities of 4 or 20 OD600. After resuspension, the cultures were transferred aseptically to anaerobic culture containers, which contained MgCO3. The containers were sparged with CO2 at 1 L/min at STP.

Small Inoculum Experiments (4 OD600). Triplicate cultures were grown aerobically using 125-mL shake flasks containing 25 mL of LB medium with appropriate antibiotic concentration. A volume of this culture was centrifuged, and the cells collected were resuspended in 18 mL of anaerobic medium to an initial OD600 of 4. The cells were transferred aseptically to 45-mL glass anaerobic tubes containing 0.5 g of MgCO3. The resuspended culture was sparged with sterile CO2 at 1 L/min STP for 8 s and rapidly capped with open top caps and PTFE/silicone rubber septa to ensure anaerobic conditions. A sample of the initial unincubated media was saved for analysis and samples were withdrawn with a syringe at 48, 96, and 168 h.

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Effect of PYC Overexpression. The current study investigates the effect of expressing the heterologous PYC in a doubly mutated strain of E. coli, SBS110MG. Anaerobic tube experiments were performed under a complete atmosphere of CO2 using an initial OD600 of 4 with strain SBS110MG with and without plasmid pHL413 to assess the effect of overexpressing the pyc gene. Samples taken at different time intervals (48, 96, and 168 h) indicated that the expression of the pyc gene (plasmid pHL413) was necessary to increase the glucose uptake and to obtain high succinate yields. Fermentations with SBS110MG(pTrc99A) and SBS110MG(pHL413) were terminated after 168 h. At this point the control strain consumed only 11% of the initial glucose added (20 g/L) with low succinate yield and high acetate yield while SBS110MG(pHL413) consumed 100% of the initial glucose achieving a succinate yield of 1.3 mol/mol.

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mol/mol to 1.3 mol/mol, while the acetate yield dropped from 1.2 mol/mol to 0.8 mol/mol. The residual formate yield was also lower in the strain overexpressing pyc relative to the control strain. The residual formate dropped from 0.7 mol/mol to 0.5 mol/mol.

Effect of fdhF Deletion. To investigate the effect of eliminating the fdhF gene, which encodes the native formate dehydrogenase (FDH) FDH-H subunit of the formate hydrogen lyase (FHL) complex that converts formate to CO₂ and H₂ (Rossmann et al., 1991; Sawers, 1994), strain SBS880MGK was constructed by eliminating the native fdhF gene from SBS110MG, both strains were transformed with pHL413 and anaerobic tube experiments were performed.

Figure 3 shows the results obtained in anaerobic tube experiments performed using an initial OD 600 of 4. The cultures were analyzed after different time intervals (48, 96, and 168 h). A comparison of the results for the strain SBS110MG(pHL413) with SBS880MGK(pHL413) indicates the effect of eliminating the native FDH on the metabolic pattern of SBS110MG(pHL413). As can be seen from Figure 3, no significant differences were observed for the first 48 h of culture between both strains in glucose consumption, succinate, acetate, residual formate levels or succinate yield. After 96 h significant differences in glucose consumption and succinate levels were noticed but no apparent change in the succinate yield was observed. After 168 h a decrease in residual formate yield was observed for SBS110MG(pHL413) relative to 48 h, while the residual formate yield remained constant for the strain lacking FDH activity. Glucose consumption, succinate levels and yield were significantly lower at this time. The acetate levels were similar, however the acetate yield was found to be higher for the fdhF⁻ strain. Strain SBS110MG(pHL413) consumed 100% of the glucose after 168 h while SBS880MGK(pHL413) consumed 62% of the initial glucose. SBS110MG(pHL413) was able to sustain the succinate molar yield in the range of 1.2–1.3 through the entire fermentation period, while the succinate yield of SBS880MGK(pHL413) dropped to 0.9 mol/mol by the end of the fermentation process. Table 2 shows the molar yields of products during the anaerobic succinate production phase for SBS110MG(pHL413) and SBS880MGK(pHL413). Succinate, residual formate or acetate yield were similar in both strains after 48 h of culture. After 96 h, acetate and residual formate yields decrease for SBS110MG(pHL413) relative to SBS880MGK(pHL413); however, the succinate yield was not significantly different. After 168 h, the residual formate and acetate yield of SBS110MG(pHL413) decreases abruptly to 0.19 and 0.8, respectively, with concomitant increase in glucose consumption and succinate levels (see Figure 3) in contrast to the strain lacking the native FDH. As expected the residual formate yield remained unchanged after each time interval analyzed with the fdhF⁻ strain.

Effect of ptsG Deletion. The presence of acetate at high concentrations is undesirable in the fermentation broth because it increases the cost of succinate purification. It is for this reason that we looked at different options to increase the succinate:acetate ratio. Another
possible option could be to inactivate the acetate pathway but only if an additional route is provided to avoid acetyl-CoA accumulation.

Previous studies revealed that when a mutation in \textit{ptsG} was introduced into \textit{E. coli} strains that could ferment glucose, the resulting strain was able to produce more succinate and less acetate (Chatterjee et al., 2001). Based on these findings and to evaluate the possibility of a further increase in succinate yield and a decrease in acetate we transferred the \textit{ptsG} mutation into strain SBS110MG to create SBS220MG. The triple mutant was transformed with plasmid pHL413 and experiments were performed under anaerobic conditions using a high cell density inoculum. The results of these experiments are depicted in Figure 2, including glucose consumed (mM) and the concentration of different metabolites produced (mM) after 48 h of culture. Pyruvate, lactate and ethanol concentrations were not detected. Inactivation of the \textit{ptsG} system significantly decreased the glucose consumed, succinate, residual formate and acetate levels by 33%, 28%, 40%, 43% respectively while increasing the succinate yield and reducing the acetate yield as expected. The percent increase/decrease in product yields of the \textit{ptsG}- strain relative to the double mutant strain SBS110MG(pHL413) and SBS880MG(pHL413) at \( P \leq 0.05 \).

Table 2. Molar Yields of Products during Anaerobic Succinate Production Phase of Various Strains\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>48 h</th>
<th>96 h</th>
<th>168 h</th>
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<tbody>
<tr>
<td><strong>strain</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SBS110MG(pHL413)</td>
<td>1.32</td>
<td>0.91</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>0.97</td>
<td>1.17</td>
</tr>
<tr>
<td>SBS880MG(pHL413)</td>
<td>1.10</td>
<td>1.04</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>0.87</td>
<td>0.92</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>1.14</td>
<td>0.92</td>
<td>1.17</td>
</tr>
</tbody>
</table>

\(^a\) Standard deviations are shown in parentheses. Asterisk (*) denotes statistically significant differences between strains SBS110MG(pHL413) and SBS880MG(pHL413) at \( P \leq 0.05 \).

Figure 3. Metabolite concentrations (mM) and succinate yield. Results from anaerobic experiments using glucose as a carbon source on LB medium with a starting OD\(_{600}\) of 4. Concentrations shown are from samples collected after 48, 96, and 168 h of culture (average of triplicate cultures). The error bars represent standard deviations.
Discussion

Genetically engineered \textit{E. coli} strains have been created to achieve high succinate yield and productivity by inactivating either \textit{ldhA} and \textit{adhE} or \textit{ldhA}, \textit{adhE} and the \textit{ptsG} system. The results described here indicate that these strains can ferment glucose to produce a succinate molar yield of 1.3 mol/mol and 1.4 mol/mol, respectively. However the double mutant, SBS110MG, deficient only in \textit{ldhA} and \textit{adhE} overexpressing PYC showed to ferment glucose much faster.

In previous studies it was found that a mutant of \textit{E. coli}, NZN111, deficient in \textit{ldhA} and \textit{pfl} genes, is blocked in the metabolism of pyruvate and fails to grow fermentatively even when the medium is supplemented with acetate for biosynthetic needs (Stols and Donnelly, 1997). However when transformed with \textit{mdh} gene encoding malate dehydrogenase from \textit{Ascaris suum}, \textit{E. coli} NZN111 grew anaerobically with a succinate yield of 0.6 mol/mol (Stols et al., 1997). Later, a spontaneous mutant of NZN111, designated as AFP111, was shown to have a mutation in the \textit{ptsG} gene (Chatterjee et al., 2001). When grown anaerobically under 5% H$_2$–95% CO$_2$, this strain produced a succinate yield of 1 mol/mol (Donnelly et al., 1998). Additionally in another study this strain grown under exclusively anaerobic conditions on rich media and an atmosphere of CO$_2$ achieved a succinate yield of 1.34 mol/mol (Vemuri et al., 2002a). Moreover AFP111 over-expressing PYC from \textit{Rhizobium etli}, first grown anaerobically for biomass generation and then subjected to anaerobic conditions under CO$_2$ resulted in a succinate yield as high as 1.46 mol/mol (Vemuri et al., 2002a) and when grown in a dual fed-batch mode a yield as high as 1.78 mol/mol was achieved (Vemuri et al., 2002b). It is worth mentioning that the dual phase fermentations performed in the latter study contained twice the amounts of tryptone and yeast than those experiments conducted exclusively under anaerobic conditions. Despite the very high yields achieved with these strains in the latter two studies, it is difficult to decouple the strain performance from the optimized culture conditions. Succinate yield can be affected by media composition and operational conditions. These factors can sometimes be confounded with strain performance. We refer to strain performance as the capacity of the organism to convert glucose to succinate optimizing the use of carbon and electrons present in this carbon source to maximize the succinate yield from the sole use of glucose. An example is the discrepancy in succinate yield observed in two different studies using the same strain, AFP111, under exclusively anaerobic growth and a headspace of CO$_2$. Chatterjee et al. (2001) reported a succinate yield from glucose obtained under anaerobic conditions of 0.92 mol/mol (0.60 g/g), while Vemuri et al. reported a yield of 1.34 mol/mol (0.88 g/g) using the same strain, also under anaerobic conditions and CO$_2$ headspace (Vemuri et al., 2002b). Optimized medium as well as improved cultivation conditions, such as the rate of oxygen transfer in the aerobic culture, the time of transition from aerobic to anaerobic and the fermentation mode, for example, fed-batch, can improve the capacity of the system to generate higher succinate yields as occurs with dual phase fermentations. Vemuri et al. (2002a) observed in their study that expression of isocitrate lyase, key enzyme from the glyoxylate shunt present during aerobic fermentation and at the onset of the anaerobic fermentation might be the responsible of higher succinate yields due to the activity of two different succinate pathways, the reductive arm of the TCA cycle and the glyoxylate shunt. Additionally they stated in their study that critical aerobic enzymatic activity might influence the anaerobic production phase in NZN111 and AFP111, therefore providing means for these organisms to sustain a redox balance under anaerobic conditions. However to have an adequate succinate production system suitable for industrial purposes it is important to develop robust strains that can be subjected to a wide range of variations in the operating conditions without any detrimental effects on its performance. Relying upon the aerobic enzymes that are produced during the aerobic phase can be risky since the lifetime of these enzymes can be as short as couple of minutes in an environment where they can be subjected to degradation by proteases and expression of these enzymes is repressed once fully anaerobic conditions are established.

One main advantage of our system compared to previously reported systems is that it constitutes a platform host, a potential candidate for further engineering to increase succinate. In our system, under fully anaerobic conditions, the presence of the PFL pathway is necessary to prevent pyruvate accumulation and also to generate both formate and acetyl-CoA. Formate can be used as a source of reducing equivalents for example by incorporating an NAD$^+$-dependent formate dehydrogenase (Berrios-Rivera et al., 2002) to generate NADH and increase succinate through the fermentative pathway. The carbon present in acetyl-CoA can be diverted somehow to the formation of succinate to prevent acetate accumulation. The base strain, SBS110MG, presented in this paper has shown to be a fast glucose consumer, easy to grow in LB medium, stable and very robust.

Further experiments are being conducted to divert acetyl-CoA toward the glyoxylate pathway to recover this carbon and generate more succinate.

Effect of PYC Overexpression. In this study, we compare a double mutant strain (\textit{ldhA}, \textit{adhE}) of \textit{E. coli}, SBS110MG, in the absence or presence of the enzyme pyruvate carboxylase under anaerobic conditions in an atmosphere of CO$_2$. In addition we evaluate the importance of the native enzyme FDH in this system and the effect of the incorporation of an additional mutation in the \textit{ptsG} system.

In wild type \textit{E. coli}, PEP carboxylase represents the main anaplerotic reaction to replenish the OAA pool. The fraction of PEP not flowing to OAA is converted to pyruvate and under anaerobic conditions for SBS110MG, high levels of acetate were observed to accumulate with an acetate yield of 1.2 mol/mol and a succinate yield of 0.2 mol/mol. Additionally, the glucose consumption of this strain was very slow due to inability to achieve a proper fermentation balance. In the absence of lactate dehydrogenase, pyruvate formate lyase is the only route to assimilate pyruvate. The acetyl-CoA produced by PFL may be converted to acetate or ethanol, but in the absence of the enzyme ADH the cells cannot regenerate the NADH produced during glycolysis and hence glucose consumption is reduced. PYC overexpression restores the ability of SBS110MG to ferment glucose, which is defective due to deletion of the two NAD$^+$-regenerating pathways as predicted by considering the redox balance. In the presence of plasmid pHL413, which encodes PYC from \textit{Lactococcus lactis}, SBS110MG is provided with an alternative route to OAA formation and at the same time, by producing succinate the cells can regenerate the NADH produced from glucose consumption. Overexpression of PYC in SBS110MG increased succinate yield from 0.2 to 1.3 mol/mol, decreased acetate from 1.2 to 0.8 mol/mol and increased glucose consumption 4-fold.
In the single adhE mutant the carbon flow is diverted to lactate with a concomitant decrease through the PFL pathway (data not shown) and in the absence of the LDH pathway, direct conversion of pyruvate to succinic acid through the PYC pathway plays an essential role to prevent redox imbalance. Succinate requires two moles of NADH per mole formed and in the absence of competing pathways for NADH oxidation; conversion to succinic acid either through PEP or PYC is the only way to regenerate NAD+.

The advantage of SBS110MG(pHL413) relative to other E. coli succinate producing systems is that the PFL pathway is preserved to allow formation of acetyl-CoA, which is used for biosynthesis, as well as a substrate for PTA for subsequent conversion to acetate by acetate kinase, accompanied by the synthesis of ATP. Through the conversion of PEP to OAA, the free energy of PEP is lost as inorganic phosphate, furthermore the conversion of pyruvate to OAA by pyruvate carboxylase also requires ATP hydrolysis, and therefore the operation of the acetate pathway can provide an additional ATP/glucose to meet the cell energy requirements. Under anaerobic conditions, the ack-pta branch enhances acetyl-CoA recycling and promotes growth; this is observed by a decrease in cell density for ack-pta mutants relative to the wild type E. coli strain (Yang, 1999). Additionally, formate generated by PFL can be used as a source of reducing equivalents, such as the use of a NAD-dependent FDH as reported previously (Berrios-Rivera et al., 2002; Berrios-Rivera et al., 2004), to further increase the succinate yield.

The effect of using different inoculum sizes did not affect the performance of the strain, at first an initial inoculum of 4 OD600 was used to perform preliminary experiments and it was decided to increase the inoculum size to 20 OD600 to decrease the fermentation time from 168 to 48 h. Succinate yield and metabolite distribution were very similar for both inoculum sizes. When a small inoculum was used, samples withdrawn at 24 h contained more than 95% of the glucose, whereas when a larger inoculum was utilized, glucose was almost completely consumed at this time interval; therefore samples had to be withdrawn at different time points for small and large inoculum experiments.

**Effect of fdhF Deletion.** Anaerobic succinate production experiments were performed with strains SBS110MG(pHL413) and SBS880MGK(pHL413) to investigate the effect of eliminating the native FDH activity on succinate yield. One significant finding of this study is the demonstration of the importance of FHL activity in succinate production. These experiments suggest that inactivation of the native FDH leads to a decrease in succinate yield when reducing equivalents become limiting in the culture. Previous studies have shown that elimination of the native FDH in wild type E. coli creates a more oxidized environment evidenced by a decrease in the ethanol-to-acetate ratio together with a decrease in succinate and lactate (Berrios-Rivera et al., 2002). It has been suggested, as well, that wild type E. coli under anaerobic conditions is capable of recapitulating some of the H2 produced from the degradation of formate (Berrios-Rivera et al., 2002). Additionally, it has been reported that the presence of H2 prevented the accumulation of fumarate (NADH is required in the conversion step of fumarate to succinate) in a succinate producing triple mutant (pfl, ldhA, ptsG) strain grown anaerobically (Vemuri et al., 2002a). The mechanism of the capturing of additional reducing equivalents is not well understood, possibly reducing equivalents are recirculated internally or perhaps hydrogen is first released and then taken up again by a hydrogenase (Clark, 1989). One of the most relevant observations was the decrease over time of the residual formate yield observed in SBS110MG(pHL413) with a simultaneous increase in succinate levels in contrast to SBS880MGK(pHL413). A possible explanation for the drop in succinate yield after 168 h for the fdhF- strain could be the depletion of reducing power from the medium. At the beginning of the fermentation process, extra reducing power could have been derived from yeast extract and tryptone present in LB medium therefore no significant differences were seen between the two strains. Depletion of the reducing power from the medium possibly forced the cells to use formate as an available source. Previous studies using a wild type E. coli strain have shown that the experimental NADH yield (a measurement of NADH availability) from glucose is slightly higher in complex medium than minimal medium supporting the hypothesis that the succinate yield is sustained during the fermentation process due to available reducing power derived from formate. (Berrios-Rivera et al., 2004). It is worth noting that FHL is induced anaerobically both by its substrate formate and by increased acidity (Clark, 1989; Sawers, 1984). It is also possible that formate consumption increases with fermentation time, when the substrate and acidity levels are increased. Previous findings revealed that inclusion of H2 gas in the fermentation atmosphere could increase succinate yield in an E. coli pfl-, ldhA- mutant strain overexpressing the NAD+-dependent malic enzyme (Stols and Donnelly, 1997). We have successfully demonstrated that E. coli can be used efficiently to generate reducing power to further increase succinate yield without inclusion of any exogenous electron donors. The remarkable efficiency of this system is evidenced by the ability of the cells to achieve proper balance between the carbon converted to succinic acid and the carbon used to derive reducing equivalents.

**Effect of ptsG Deletion.** The effect of an additional mutation in the ptsG system of strain SBS110MG(pHL413) was examined by creation of strain SBS220MG. This strain was subsequently transformed with plasmid pHIL413 for comparison with SBS110MG(pHL413) in terms of the metabolic pattern. As observed from Figure 3, the succinate yield increased by only 7% while the glucose consumption and acetate yield were reduced by 33% and 15% respectively. Previous studies showed that single ptsG E. coli mutants have lower glucose uptake rates than wild type E. coli as well as lower specific acetate yields under aerobic conditions (Chou et al., 1994). The triple mutant culture, SBS220MG, followed the same trend as the latter study. Furthermore other studies have also shown that a mutation in ptsG shifted the fermentation metabolism toward succinate with a decrease in acetate excretion (Chatterjee et al., 2001). The product of ptsG, the glucose-specific PTS permease EIICBα, catalyzes the transport and phosphorylation of glucose using phosphate derived from PEP. In wild type E. coli, 1 mol of PEP per mole of glucose transported is converted to pyruvate, and in the absence of an enzyme capable of diverting pyruvate toward succinate, the pyruvate will be committed to either PFL or LDH pathways. It is for this reason that inactivation of EIICBα causes in principle an increase in the PEP pool, favoring succinate generation. In the presence of PYC and an efficient conversion to products in a system with a functional PTS, the ptsG mutation has little effect since pyruvate can be redirected toward OAA also favoring succinate yield. This affirmation is supported by the fact that the ptsG mutant strain SBS220MG (pHL413) did not
show a striking increase in succinic acid, whereas a 33% reduction in glucose consumption was obtained instead.

**Conclusion**

In this study, we have developed a high yield anaerobic succinate production system capable of producing succinate at a yield higher than the maximum theoretical value of 1.0. In this system, the overexpression of heterologous PYC enhanced the ability of SBS110MG to utilize glucose and succinate yield increased from 0.2 mol/mol to 1.3 mol/mol and acetate yield decreased from 1.2 mol/mol to 0.8 mol/mol. The current system also provides a platform for further improvement by conserving the PFL pathway that provides acetyl-CoA and an additional potential NADH source in the form of formate. Future studies will be conducted to divert the carbon present in acetyl-CoA away from acetate and to generate more succinate.

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**References and Notes**


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