Redistribution of Metabolic Fluxes in the Central Aerobic Metabolic Pathway of *E. coli* Mutant Strains with Deletion of the *ackA-pta* and *poxB* Pathways for the Synthesis of Isoamyl Acetate

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Although the bacterium *E. coli* is chosen as the host in many bioprocesses, products derived from the central aerobic metabolic pathway often compete with the acetate-producing pathways *poxB* and *ackA-pta* for glucose as the substrate. As such, a significant portion of the glucose may be excreted as acetate, wasting substrate that could have otherwise been used for the desired product. The production of the ester isoamyl acetate from acetyl-CoA by ATF2, a yeast alcohol acetyl transferase, was used as a model system to demonstrate the beneficial effects of reducing acetate production. All strains tested for ester production also overexpressed *panK*, a native *E. coli* gene that previous studies have shown to increase free intracellular CoA levels when fed with pantothenic acid. A recombinant *E. coli* strain with a deletion in *ackA-pta* produces less acetate and more isoamyl acetate than the wild-type *E. coli* strain. When both acetate-producing pathways were deleted, the acetate production was greatly reduced. However, pyruvate began to accumulate, so that the overall ester production remained largely unchanged. To produce more ester, a previously established strategy of increasing the flux from pyruvate to acetyl-CoA was adopted by over-expressing pyruvate dehydrogenase. The ester production was then 80% higher in the *poxB, ackA-pta* strain (0.18 mM) than that found in the single *ackA-pta* mutant (0.10 mM), which also overexpressed PDH.

**Introduction**

Metabolic engineering utilizes recombinant DNA technologies to manipulate metabolic pathways for increased yield of desired products. Applications of metabolic engineering have expanded in both academic and industrial settings (Bailey, 1991; Cameron and Chaplen, 1997; Lee and Papoutsakis, 1999; Stephanopoulos et al., 1998; Stephanopoulos and Vallino, 1990). Because of the abundance of genetic information, the ease of genetic manipulation, and the simplicity of culture cultivation, *E. coli* is a widely used model system for metabolic engineering and recombinant protein production. Several valuable products are directly or indirectly derived from the central metabolic pathway (Figure 1) in *E. coli*, using glucose as substrate.

A significant portion of the aerobic metabolic flux of *E. coli* is channeled to acetate under conditions of excess glucose. When the cells are not under starvation, they uptake excess glucose beyond that utilized for biomass synthesis and therefore excrete the carbon source in the form of acetate (Doelle et al., 1981; Farmer and Liao, 1997; Kleman and Strohl, 1994). Because of the abundance of genetic information, the ease of genetic manipulation, and the simplicity of culture cultivation, *E. coli* is a widely used model system for metabolic engineering and recombinant protein production. Several valuable products are directly or indirectly derived from the central metabolic pathway (Figure 1) in *E. coli*, using glucose as substrate.

Two major acetate-producing pathways active aerobically in *E. coli* are pyruvate oxidase (*poxB*) and acetate kinase/phosphotransacetylase (*ackA-pta*). Previous studies have analyzed strains in which the *ackA-pta* pathway was deleted (Chang, et al., 1999; Hahm et al., 1994; Kakuda et al., 1994a; Kakuda et al., 1994b; Tomar et al. 2003; Yang et al., 1999a; Yang et al., 1999b). The *ackA-pta* pathway is active both aerobiocally and anaerobically to convert acetyl-CoA to acetate (Hahm et al., 1994; Yang et al., 1999a). Under aerobic conditions, *ackA-pta* strains can still convert glucose to acetate through the *poxB* pathway, which decarboxylates pyruvate to acetate and carbon dioxide.

This current report analyzes the metabolic behavior of strains containing a mutation in *ackA-pta, poxB*, or
both. We use the biosynthesis of the ester isoamyl acetate from acetyl-CoA as a model system to demonstrate the beneficial effects of reducing acetate production through deletion of both the ackA-pta and poxB pathways. Isoamyl acetate is a valuable chemical used as an industrial solvent, plasticizer, cleaner, and a solvent for lacquer coatings and nail polish. However, its most important use is in the food industry where 74,000 kg/year are used (Krishna et al., 2001). Microbial fermentation is considered as an alternative route for “natural” production of esters.

Materials and Methods

Bacterial Strains and Plasmids. Strains and plasmids used in this work are listed in Table 1. MG1655 served as a control strain, and a parent for construction of the three mutants CD58, CD61, and CD6158. CD58 (MG1655, poxB) and CD61 (MG1655, ackA-pta) were created by one-step chromosomal inactivation followed by removal of the drug resistance marker (Datsenko and Wanner, 2000; Yu et al., 2000). CD6158 (MG1655, ackA-pta, poxB) was created by P1 transduction of the host strain CD61 with lysate from CD58. All of the plasmids listed have been previously reported.

Strain Characterization. Luria—Bertani broth supplemented with 20 g/L of glucose was used for all strain characterizations. A 15-mL culture (initial OD600 = 0.1) was grown in 125-mL Erlenmeyer flasks. The flasks were capped with foam stoppers. They were grown in an orbital shaker at 250 rpm and 37 °C for 24 h. At the end of the experiment, the cultures were analyzed for metabolite production.

Isoamyl Acetate Study. Luria—Bertani broth with 20 g/L of glucose was further supplemented with final concentrations of 5 mM pantethic acid, 10 mM isomyl alcohol, and 1 mM IPTG. In addition, antibiotics were appropriately added to select for the desired plasmid(s): 100 mg/L kanamycin and 1 g/L ampicillin. A 10-mL culture (initial OD600 = 0.5) was grown in 250-mL Erlenmeyer flasks. The flasks were capped with foam stoppers to prevent the release of the volatile compounds isoamyl acetate and isoamyl alcohol. They were grown in an orbital shaker at 250 rpm and 37 °C for 24 h. At the end of the experiment, the cultures were analyzed for metabolite and isoamyl acetate production.

Quantification of Extracellular Metabolites. A 1-mL sample of cell culture broth was centrifuged at 8000g for 3 min. The supernatant was filtered through a 0.45-μm syringe filter for HPLC analysis and stored frozen until analyzed. Metabolites such as residual glucose, acetate, ethanol, lactate, and pyruvate were quantified using an HPLC system (Shimadzu-10A systems, Shimadzu, Columbia, MD) equipped with a cation-exchange column (HPX-87H, BioRad Labs; Hercules, CA), a UV detector (Shimadzu SPD-10A), and a refractive index detector (Waters 2410, Waters, Milford, MA). Pyruvate was quantified using a UV detector, and the other metabolites using a refractive index detector. A 2.5 mM H2SO4 solution was used as the mobile phase at a flow rate of 0.6 mL/min. The HPLC column was operated at 55 °C.

Quantification of Isoamyl Acetate. Isoamyl acetate was determined by headspace gas chromatography, a protocol modified from Vadali et al. (2004c). The flask was heated at 50 °C for 30 min, and 1 mL of headspace gas was injected via a gastight syringe into a Hewlett-Packard 6890 series gas chromatograph equipped with an Alltech 6′ × 0.25" × 2 mm Poropak QS 80/100 column at a static temperature of 220 °C for 25 min. The injector and detector temperatures were 215 and 245 °C, respectively. A 6% ethyl acetate solution was used as an internal standard to correct for pressure differences.

Results

Strain Characterization. Mutations in ackA-pta and/ or poxB were created by one-step chromosomal inactivation (Datsenko and Wanner, 2000), and cultures of the strains were then characterized using HPLC analysis. Genomic PCR with appropriate primers was used to demonstrate the proper chromosomal modification and thus define the genotype. As shown in Table 2, the mutation(s) affected the overall growth of the strains, most notably in the double mutant. The ackA-pta, poxB double mutant had a final OD600 that was about 70% that of the wild type. This difference in growth characteristics can also be seen in the amount of glucose consumed (Table 2): CD6158 consumed 37% less glucose than MG1655.

Deleting either acetate-producing pathway does not significantly decrease the amount of acetate produced after 24 h. As seen in Figure 2, deleting the ackA-pta pathway only reduces acetate production by 4%, and deleting the poxB pathway reduces acetate production by 10%, as compared to the wild type. Deleting both acetate-producing pathways, however, significantly reduces the final concentration of acetate to only 7% of that of the wild type. The molar acetate yields followed a similar pattern (Figure 2).

Cultures of the strain with an inactivated ackA-pta pathway exhibited no extracellular pyruvate after 24 h, but cultured strains with poxB inactivation showed an

<table>
<thead>
<tr>
<th>genotype</th>
<th>strains</th>
<th>references</th>
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<tbody>
<tr>
<td>MG1655</td>
<td>parent (F− λ−)</td>
<td>ATCC 47076</td>
</tr>
<tr>
<td>CD58</td>
<td>poxB</td>
<td>this study</td>
</tr>
<tr>
<td>CD61</td>
<td>ackA-pta</td>
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<td>CD6158</td>
<td>poxB, ackA-pta</td>
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Table 1. Strains and Plasmids Used

<table>
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<tr>
<td>CD6158</td>
<td>poxB, ackA-pta</td>
<td>this study</td>
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Table 2. Optical Density and Glucose Consumed by Cultures of Strains

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<tr>
<th>strains</th>
<th>OD600</th>
<th>glucose consumed (mM)</th>
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<tbody>
<tr>
<td>MG1655</td>
<td>5.8 ± 0.3</td>
<td>24.4 ± 0.2</td>
</tr>
<tr>
<td>CD58</td>
<td>4.8 ± 0.1</td>
<td>28.7 ± 0.1</td>
</tr>
<tr>
<td>CD61</td>
<td>5.7 ± 0.1</td>
<td>26.7 ± 1.0</td>
</tr>
<tr>
<td>CD6158</td>
<td>4.3 ± 0.1</td>
<td>15.5 ± 1.7</td>
</tr>
</tbody>
</table>

a The strains MG1655, CD58, CD61, CD6158 were grown under aerobic conditions. The strains were grown in LB + 20 g glucose/L medium. The cultures were sampled 24 h after inoculation. The data shown are means ± standard deviation of three replicate experiments.
accumulation of pyruvate (Figure 2). Deleting both acetate pathways results in an even greater excretion of pyruvate, almost four times that of the popB mutant.

**Isoamyl Acetate with CoA Manipulation.** Previously we have observed that increasing the free CoA levels by overexpressing pantothenate kinase \((\text{panK})\) with pantothenic acid supplementation leads to an increase in isoamyl acetate (Vadali et al., 2004a). We have also shown that deleting the \(\text{ackA-pta}\) pathway, which competes with ester-producing genes for acetyl-CoA, increases the ester produced (Vadali et al., 2004c). In this study, we examine the effect that deleting \(\text{popB}\) in an \(\text{ackA-pta}\) mutant host strain has on isoamyl acetate production. To test our hypothesis, we constructed two strains that overexpress \(\text{panK}\), CD61(pATCA) and CD6158(pATCA). Both strains also overexpress alcohol acetyltransferase (AAT) and produce isoamyl acetate when isoamyl alcohol is added to the cell culture medium. Results from aerobic shake flask experiments (Figure 3a) showed no significant difference between CD61(pATCA) and CD6158(pATCA) in the amount of isoamyl alcohol produced.

**Metabolite Characterization of Isoamyl Acetate-Producing Strains.** Two more recombinant strains, CD6158(pATCA, ptac-85) and CD6158(pATCA, pGS367), were constructed to assist a study of metabolic distribution in CD6158 with PDH overexpression. Although both strains overexpress pantothenate kinase, the flux to free intracellular CoA will not increase without the addition of pantothenic acid (Vadali et al., 2004a). Both strains also overexpress alcohol acetyltransferase. Only CD6158(pATCA, pGS367) has PDH overexpressed; CD6158(pATCA, ptac-85) carries the control plasmid for pGS367.

As seen in Table 3, overexpressing pyruvate dehydrogenase allows the cells to grow to a higher optical density. Adding pantothenic acid to either strain increases the amount of glucose consumed.

**Figures and Tables:***

*Figure 2.* Analysis of extracellular metabolites of acetate pathway mutant strains: (a) acetate and pyruvate levels, (b) extracellular metabolite molar yields. The strains MG1655, CD58, CD61, and CD6158 were grown in LB + 20 g glucose/L medium under aerobic conditions. The cultures were sampled 24 h after inoculation. The data shown are means ± standard deviation of three replicate experiments.

*Figure 3.* Production of isoamyl acetate: the levels of isoamyl acetate are shown in the strains (a) CD61(pATCA) and CD6158(pATCA, ptac-85) and (b) CD61(pATCA, pGS367) and CD6158(pATCA, pGS367), grown under aerobic conditions at 37 °C. The strains were cultured in LB, 2% glucose, 1 mM IPTG, 10 mM isoamyl alcohol, 100 mg/L kanamycin, 5 mM pantothenic acid (PA), and 1 g/L ampicillin (for part b only). The cultures were sampled 24 h after inoculation for ester production. The data shown are means ± standard deviation of three replicate experiments.

*Table 3.* Optical Density and Glucose Consumed

<table>
<thead>
<tr>
<th>Strains</th>
<th>OD</th>
<th>Glucose Consumed (mM)</th>
</tr>
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<tbody>
<tr>
<td>CD6158(pATCA, ptac-85) + PA</td>
<td>5.5 ± 0.1</td>
<td>23.1 ± 0.1</td>
</tr>
<tr>
<td>CD6158(pATCA, pGS367) + PA</td>
<td>11.3 ± 0.1</td>
<td>47.1 ± 0.2</td>
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<tr>
<td>CD6158(pATCA, ptac-85) − PA</td>
<td>6.3 ± 0.1</td>
<td>36.0 ± 1.8</td>
</tr>
<tr>
<td>CD6158(pATCA, pGS367) − PA</td>
<td>11.3 ± 0.1</td>
<td>50.9 ± 2.5</td>
</tr>
</tbody>
</table>

*The strains CD6158(pATCA, ptac-85) and CD6158(pATCA, pGS367) were grown in LB, 2% glucose, 1 mM IPTG, 10 mM isoamyl alcohol, 100 mg/L kanamycin, and 1 g/L ampicillin, ±5 mM pantothenic acid (PA) under aerobic conditions. The cultures were sampled 24 h after inoculation. The data shown are means ± standard deviation of three replicate experiments. Without the addition of pantothenic acid (Table 3). Adding pantothenic acid to either strain increases the amount of glucose consumed.*

As Figure 4 shows, overexpressing both PDH and \(\text{panK}\), along with pantothenic acid supplementation, reduces the amount of extracellular pyruvate present in the cell culture medium after 24 h. The acetate levels do not vary significantly between the four cultures.

**Isoamyl Acetate with CoA Manipulation and PDH Overexpression.** We further examined the effect of increasing acetyl-CoA flux by overexpressing both PDH and \(\text{panK}\) (Vadali et al., 2004b), along with AAT, the ester-forming enzyme, in host strains CD61 and CD6158 by constructing CD61(pATCA, pGS367) and CD6158(pATCA, pGS367). In an aerobic shake flask experiment, CD6158(pATCA, pGS367) produced 75% more isoamyl acetate than CD61(pATCA, pGS367) (Figure 3).

**Discussion**

**Strain Characterization.** Inactivating either acetate pathway reduces the final culture density of the mutant strain with respect to the wild type, and deleting both pathways reduces the final cell density of the double mutant strain even further (Table 2). Both acetate producing pathways have been shown previously to be important to the overall fitness of \(E. \ coli\). The \(\text{ackA-pta}\)
pathway is thought to be important for two reasons: it provides a source of ATP and it helps maintain a sufficient level of acetyl phosphate, which is especially important under conditions of starvation (Nystrom and Gustavsson, 1998). Acetyl phosphate is thought to be the donor for the phosphorylation of many signal transduction regulators including CheY, PhoB, NR1, and OmpR (Chang et al., 1999; McCleary and Stock, 1994). Pyruvate oxidase was once thought to be a nonessential enzyme (Chang et al., 1994) but has recently been shown to contribute to the overall aerobic growth efficiency of E. coli (Abdel-Hamid et al., 2001).

The single mutants seem to divert acetate production to the remaining acetate-producing pathway to the extent that acetate reduction is minimal, as compared to the wild type. The strains may compensate for the loss of one pathway by diverting more glucose to the other pathway. Deleting both acetate pathways reduces the final acetate concentration drastically to only 7% of the concentration in the wild-type broth. This small but still present amount of acetate can be attributed to a number of lesser acetate-producing pathways including, but not limited to, acetylornithine deacetylase (Javid-Majd and Blanchard, 2000) and citrate lyase (Kakuda et al., 1994b).

Deleting the poxB pathway appears to lead to a bottleneck at the pyruvate node. The strain takes up glucose at a similar rate as the wild type does, but the activity of pyruvate dehydrogenase is not sufficient to channel the pyruvate to acetyl-CoA. Instead, the cell excretes the pyruvate, an effect which is even more apparent in the double mutant. These results are similar to the results previously reported (Causey et al., 2004). Wild-type E. coli generates only acetate as a byproduct. However, with the two major acetate-producing pathways deleted, the strain continues to uptake an excess of glucose, and this bottleneck is seen at the pyruvate node.

Metabolite Characterization of Isoamyl Acetate-Producing Strains. Increasing the flux of pyruvate to acetyl-CoA by overexpressing pyruvate dehydrogenase and increasing the level of available intracellular CoA has been previously shown to increase the amount of ester produced (Vadali et al., 2004b). Because deleting both poxB and ackA-pta leads to a substantial pyruvate accumulation, the strategies of knocking out the acetate pathways and increasing the flux of pyruvate to acetyl-CoA were combined in the effort to increase isoamyl acetate production. Increasing the flux of pyruvate to acetyl-CoA was accomplished by both increasing the free CoA levels via panK overexpression and by overexpressing PDH (Vadali et al. 2004b).

Isoamyl Acetate Synthesis with CoA Manipulation and PDH Overexpression. Increasing the flux of pyruvate to acetyl-CoA by overexpressing both pyruvate dehydrogenase and pantothenate kinase was a very successful strategy to increase isoamyl acetate synthesis. Ester synthesis was increased by 75% in the double mutant as compared to that of the ackA-pta mutant when AAT, PDH, and panK were all overexpressed. This result illustrates the need to increase the turnover at the pyruvate node when the acetate pathways are deleted.

Conclusion

The acetate-producing pathways poxB and ackA-pta in E. coli often compete with other desirable pathways for metabolite or protein production. Acetate production is usually not desired and is a detriment to the cells. We chose the ester isoamyl acetate, produced from acetyl-CoA by expression of ATF2, as our model system to
demonstrate the benefits of deleting the acetate pathways. Deleting the ackA-pta pathway still leaves acetate as a primary product. Deleting poxB alone also results in high levels of acetate excretion. Inactivating both pathways reduces the amount of extracellular acetate significantly, but the double mutant strain excretes large amounts of pyruvate instead. The ackA-pta/poxB mutant strain did not synthesize a significantly different concentration of isovaleryl acetate than the ackA-pta single mutant strain when both strains overexpressed pantothenate kinase and alcohol acetyltransferase. The double mutant strain channeled less glucose to acetate than the single mutant strain, but it instead excreted pyruvate. With pyruvate dehydrogenase overexpressed in both of these strains, less pyruvate was excreted, and a higher concentration of isovaleryl acetate was synthesized.

Acknowledgment

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References and Notes


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