Metabolic Engineering of Klebsiella oxytoca M5A1 for Ethanol Production from Xylose and Glucose†

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The efficient diversion of pyruvate from normal fermentative pathways to ethanol production in Klebsiella oxytoca M5A1 requires the expression of Zymomonas mobilis genes encoding both pyruvate decarboxylase and alcohol dehydrogenase. Final ethanol concentrations obtained with the best recombinant, strain M5A1 (pLOI555), were in excess of 40 g/liter with an efficiency of 0.48 g of ethanol (xylose) and 0.50 g of ethanol (glucose) per g of sugar, as compared with a theoretical maximum of 0.51 g of ethanol per g of sugar. The maximal volumetric productivity per hour for both sugars was 2.0 g/liter. This volumetric productivity with xylose is almost twice that previously obtained with ethanologenic Escherichia coli. Succinate was also produced as a minor product during fermentation.

Pentose sugars are abundant in nature as a major component of lignocellulosic biomass. These sugars represent a potential resource for the production of fuel ethanol (23). However, no naturally occurring microorganisms have been found which rapidly and efficiently ferment pentoses to high levels of ethanol (13, 26). One approach to solving this problem has been to transfer genes encoding the ethanol pathway in Zymomonas mobilis (pdc and adhB) into Escherichia coli and to express appropriate levels of pyruvate decarboxylase (PDC) and alcohol dehydrogenase II (ADHII) (1, 10–12, 18). This metabolic engineering of E. coli has produced recombinants capable of rapid fermentation to ethanol of all sugars which are constituents of biomass (20, 21).

In principle, the transfer of Z. mobilis genes to other organisms could be used to produce recombinants with additional useful traits for ethanol production. Recombinants of Erwinia chrysanthemi (28) and Klebsiella planticola (8, 29) have been constructed by using the Z. mobilis pdc gene alone. However, pyruvate metabolism was completely diverted to ethanol as a product of fermentation. Under selected conditions, ethanol yields per gram of sugar utilized were quite good, although the final levels of ethanol achieved were low. Low levels of ethanol were also produced by E. coli recombinants expressing only the pdc gene (3). Problems associated with these pdc recombinants included plasmid instability, decreased ethanol tolerance (which appeared to limit final product concentrations), decreased growth rate under fermentation conditions, decreased cell yield, incomplete conversion of substrate, and accumulation of acidic fermentation products.

Two factors were identified as contributing to the poor performance of the original K. planticola recombinant containing pZM15(pdc) (29). The low expression of PDC from pZM15 was proposed as being insufficient to divert pyruvate metabolism. Higher ethanol yields were subsequently obtained with constructs which expressed higher levels of PDC (8). The accumulation of organic acids (formate and acetate) was proposed to contribute to increased ethanol toxicity. Formate and acetate production was blocked by the selection of K. planticola pyruvate formate-lyase mutants (8). Although the elimination of this competing fermentation pathway improved ethanol production, the performance of these mutants remained below that observed previously with E. coli recombinants expressing both the Z. mobilis pdc and adhB genes (21).

Recombinants which contain the pdc gene alone are dependent on endogenous levels of native ADHs to couple the reduction of acetaldehyde to the oxidation of NADH. Since ethanol is only one of several abundant fermentation products normally produced by these enteric bacteria (19), it seemed possible that a deficiency in ADH (and accumulation of NADH) could contribute to the adverse effects of pdc on growth and ethanol tolerance. In this study, we have investigated the expression of both enzymes from the Z. mobilis ethanol pathway (pdc and adhB products) on ethanol production by a related enteric organism, Klebsiella oxytoca M5A1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains and plasmids used in this study are listed in Table 1. Taxonomic methods for Klebsiella species identification were used as recommended in Bergey's Manual of Systematic Bacteriology (22).

Strains were subcultured on Luria agar plates with no added sugar (15) unless they harbored plasmids encoding Z. mobilis genes. Recombinants containing adhB and pdc require a fermentable carbohydrate for survival and were maintained on plates containing 2% glucose or xylose (21). Antibiotic concentrations were as follows: ampicillin, 50 μg/ml; chloramphenicol, 40 μg/ml; and tetracycline, 12.5 μg/ml. Expression of Z. mobilis ADHII in recombinants was screened by using aldehyde indicator plates (7).

Genetic procedures and recombinant techniques. Standard procedures were used for plasmid preparations, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis (25). E. coli TC4 (6) was used as the host for all plasmid constructions.

Plasmid constructions. Since M5A1 is relatively resistant to penicillin and its derivatives, we have constructed E. coli shuttle vectors carrying the cat (Cm') or tet (Tc') genes. A tet gene was added to pLOI276 containing Z. mobilis pdc by

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inserting a 2.6-kbp EcoRI fragment from pcos2EMBL (24) into the SaI site of pLOI276. Cohesive ends were removed by treatment with the Klenow fragment of E. coli DNA polymerase before ligation. The resulting construct was confirmed by restriction analysis and designated pLOI560.

Preliminary studies indicated that E. coli B (ATCC 11303) harbored cryptic low-copy-number plasmids. A new and useful vector was constructed in vivo by randomly integrating the Z. mobilis pdc and adhB along with cat into these plasmids. This was done by isolating a 4.6-kbp promoterless PstI fragment containing pdc, adhB, and cat from pLOI510 (21). No replication functions are present on this fragment. After being circularized by self-ligation, this fragment was transformed into E. coli B with selection for Cm' on Luria agar plates containing 2% glucose. Transformants were tested on aldehyde indicator plates, and dark-red clones were selected for high-level expression of the adhB gene. Plasmid preparations from these strains were tested for their ability to transfer antibiotic resistance and Z. mobilis genes into E. coli TC4 by transformation. All recombinants were sensitive to ampicillin, indicating a lack of the pUC18 fragment containing bla and the colEl replicon. One of these, pLOI555 (8.4 kbp), produced the most intensely red colonies on aldehyde indicator plates, conferred excellent ethanol production ability to E. coli, and appeared to be present in low copy number on the basis of yields from small-scale plasmid isolations. This plasmid was used to transform K. oxytoca M5A1 with selection for Cm'.

**Plasmid stability in M5A1.** Cells harboring pLOI555, pLOI297, or pLOI560 were serially transferred in Luria broth containing 10% glucose without antibiotics for more than 60 generations at 30°C. Appropriate dilutions of cultures were plated on Luria agar containing 2% glucose without antibiotics. Colonies were tested on aldehyde indicator plates for retention of the ethanol production genes from Z. mobilis and for resistance to appropriate antibiotics.

**Fermentation experiments.** Fermentations were carried out in Luria broth containing 10% (wt/vol) glucose or xylose at 30°C and pH 6.0 with 100-rpm agitation as previously described (21). Inocula were grown overnight at 30°C from isolated colonies in unshaken flasks. Fermentations were inoculated to an initial optical density at 550 nm of 1.0 (330 mg [dry weight] of cells per liter). A Bausch & Lomb Spectronic 70 spectrophotometer was used to monitor growth.

Ethanol concentrations were determined by gas-liquid chromatography (1). Conversion efficiencies were corrected for volume changes caused by the addition of base and assumed that all sugar had been metabolized. The maximum theoretical yield of ethanol from xylose and glucose was calculated to be 0.51 g of ethanol per g of sugar, with the balance as carbon dioxide. Volumetric productivities were estimated from the most active periods and represent maximum values. All fermentation data in the tables and figures are averages from two or more batch fermentations.

**Isolation of cytoplasmic protein fraction.** Cells were grown for 24 h under the conditions of the fermentation experiments, chilled to 0°C, harvested by centrifugation (7,000 × g for 10 min), washed twice with one-third volume of 5 mM sodium phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol, and stored frozen at −20°C. Cell pellets were resuspended in an equal volume of buffer and broken by two passages through a French pressure cell at 20,000 lb/in². Membranes were removed by centrifugation at 4°C for 90 min at 100,000 × g. The supernatant containing soluble cytoplasmic proteins was stored at −70°C.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cytoplasmic proteins.** DName I-treated protein extracts were separated with a Mini-Protein II electrophoresis unit (Bio-Rad Laboratories, Richmond, Calif.) by using 10% acrylamide (14, 21). Protein was estimated by using the Bradford reagent (2). Approximately 10 μg was loaded into each lane. Molecular weight markers (Low Molecular Weight Calibration Kit; Pharmacia, Piscataway, N.J.) were included in a separate lane (phosphorylase b, 94,000; albumin, 67,000; ovalbumin 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; and o-lactalbumin 14,400). Proteins were stained with Coomassie blue R-250.

**Assay of PDC activity.** PDC activity in heat-treated French press cell extracts (see above) was measured as described previously (6). Heat treatment was used to inactivate competing native enzymes which may complicate measurements of PDC activity in recombinants.

**Analysis of volatile and nonvolatile acids.** Samples were removed for organic acid analyses after 96 h of fermentation and analyzed for acetic, lactic, and succinic acids as previously described (21). Butanediol was determined essentially as described for ethanol, except that column and injection temperatures were increased to 190°C.

**RESULTS**

**Taxonomic position of strain M5A1.** The Klebsiella strain used in this study was previously designated as Klebsiella pneumoniae M5A1 (16). This strain, a dinitrogen-fixing organism, was originally identified as Aerobacter aerogenes but renamed on the basis of antigenic properties. Since new taxonomic criteria for Klebsiella pneumoniae were defined in Bergey’s (4, 22), the species to which this strain belonged was investigated further. Strain M5A1 grew at 10°C in glucose minimal medium but failed to produce gas from lactose at 44.5°C. This strain was indole positive and utilized both m-hydroxybenzoate and gentisate as sole sources of carbon for growth at both 30 and 37°C. On the basis of these tests, M5A1 was designated K. oxytoca.

**Plasmid stability in M5A1.** Excessive instability of Z. mobilis genes in pBR322-based vectors was reported previously for K. plantaeca (29) and would not be acceptable for industrial processes. As shown in Table 2, the two pUC18-based vectors, pLOI560 and pLOI297, which also contain colEl replicons were unstable in M5A1. Only a small fraction of the population retained these plasmids after cultivation for 60 generations in the absence of antibiotic selection. In contrast, recombinants harboring pLOI555 were very stable, with 98% of the population retaining both the antibiotic resistance gene and the genes from Z. mobilis. Although only adhB expression is detected by aldehyde indicator plates, these recombinants also retained the large-colony
TABLE 2. Plasmid stability and pdc expression in recombinant strains of K. oxytoca M5A1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>PDC activity after 24 h (U/mg of protein)</th>
<th>% retaining traits* (no. of generations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLOI555</td>
<td>3.6</td>
<td>100 (38.5), 98 (68.5)</td>
</tr>
<tr>
<td>pLOI297</td>
<td>28.0</td>
<td>52 (38.6), 10 (68.6)</td>
</tr>
<tr>
<td>pLOI560</td>
<td>27.0</td>
<td>97 (32.9), 0 (62.0)</td>
</tr>
</tbody>
</table>

*Cells were sampled twice during cultivation. The aldehyde trait and antibiotic resistance were lost concurrently: Cm' for pLOI555; Tc' for pLOI297; and Tc' for pLOI560.

Expression of Z. mobilis genes in M5A1. Three plasmid constructs containing the Z. mobilis gene encoding PDC were transformed into M5A1 (Table 2). The levels of PDC were eightfold higher with the two pUC-based constructs (pLOI297 and pLOI560) than with pLOI555. Assuming a maximum specific activity for pure PDC of 100 U, this enzyme makes up more than 25% of the cytoplasmic protein in M5A1(pLOI297) and M5A1(pLOI560) and 3.6% in M5A1(pLOI555).

The expression of Z. mobilis genes in M5A1 was further confirmed by SDS-PAGE (Fig. 1). Bands which contain PDC and ADHII were easily identified by comparison with the native strain (Fig. 1). The band containing PDC is much larger in pUC-based recombinants than in M5A1(pLOI555), consistent with measurements of enzymatic activity. Although ADHII is less abundant than PDC, the relative expression of this Z. mobilis gene is also higher in M5A1 (pLOI297) than in M5A1(pLOI555). No band corresponding to ADHII is evident in M5A1(pLOI560), which contains only the Z. mobilis pdc gene.

The copy number of pLOI555 was estimated to be less than 1/10 that of the other constructs on the basis of yields in small-scale plasmid preparations. Although this estimate is only approximate, it is clear that the high levels of PDC present in the pUC-based constructs are due in part to higher copy number.

Ethanol production from xylose and glucose. Figure 2A and C and Table 3 compare the effects of Z. mobilis pdc and pdc plus adhB on ethanol production from glucose and xylose to ethanol production by the native strain M5A1. Recombinants containing the pdc gene alone produced more than twice the parental level of ethanol. Recombinants containing both pdc and adhB produced ethanol more rapidly and with higher efficiency than did M5A1(pLOI560) containing pdc alone. M5A1(pLOI555) was clearly the best construct for ethanol production, with maximum volumetric productivities of 2.1 g/liter/h for both glucose and xylose. With either sugar, this recombinant produced approximately 37 g of ethanol per liter after 30 h. Fermentation of these sugars was essentially completed after 48 h, with 45 g of ethanol per liter.

Under pH-controlled growth conditions, the addition of pdc alone reduced the maximal cell density and rate of growth by more than 50% compared with those of the native strain (Fig. 2B and D; Table 3). Growth was also limited in M5A1(pLOI297) containing a complete Z. mobilis ethanol pathway. As with ethanol production, the growth of M5A1 (pLOI555) was clearly superior. Growth of this recombinant was almost equivalent to that of the parental strain. However, unlike the parental strain, cell density progressively declined after reaching a maximum at 15 h. This decline may reflect a reduction in refractivity as ethanol accumulated, since lysis was not evident. Without the addition of base to control pH (data not shown), recombinants containing both pdc and adhB grew to more than twice the density of the parent organism as a result of a reduced rate of acid production (higher proportion of neutral fermentation products), as observed previously with E. coli (10).

Organic acid production. Despite the efficient production of ethanol by some recombinants of strain M5A1, of pdc and adhB, they were also formed by all recombinants, as evidenced by the consumption of base to maintain pH (Table 3). All recombinants produced higher levels of fermentation products from xylose than did the parent (Table 4). Acetate was the dominant acid fermentation product in the parental strain, whereas the dicarboxylic acid succinate was more abundant in the recombinant containing pdc alone (pLOI560) and in recombinants containing the complete Z. mobilis pathway. Base in excess of that needed to neutralize the amplified acidic products was consumed by the parent organism (190 mmol of KOH per liter), and this additional base may serve to neutralize formate, which was not measured. In contrast, recombinants harboring pLOI560, pLOI297, and pLOI555 produced more acid than could be neutralized by the base consumed (70, 90, and 110 mmol of KOH per liter, respectively). Analogous recombinants of E. coli catabolize complex nutrients and produce ammonia (20, 21). A similar catabolisim of complex nutrients by M5A1 recombinants may contribute natural buffers to maintain pH and reduce base consumption.

FIG. 1. SDS-PAGE gel comparing soluble proteins from recombinant strains of K. oxytoca M5A1. Molecular weights and marker proteins are shown in lane 5 and indicated on the right (in thousands). Arrows mark the bands which contain Z. mobilis PDC (a) and ADHII (b) proteins. Lanes: 1, control without plasmid; 2, pLOI555; 3, pLOI560; 4, pLOI297.

DISCUSSION

Our best recombinant strain, M5A1(pLOI555), contained both Z. mobilis pdc and adhB genes and fermented xylose to ethanol more effectively than did the best previously reported strains of E. coli (21). The maximal volumetric productivities (2.1 g of ethanol per liter per h) are almost double those of E. coli recombinants, and similarly high efficiencies and final ethanol concentrations are maintained. Unlike E. coli (20), M5A1(pLOI555) ferments xylose and glucose at equivalent rates. Plasmid pLOI555 was stably maintained in M5A1 in the absence of antibiotic selection. Since the range of substrates for M5A1 is equivalent to that of E. coli, M5A1 recombinants offer a distinct and unexpected advantage for ethanol production.
Our studies have confirmed previous reports (3, 8, 28, 29) of poor ethanol production by enteric recombinants which contain only the Z. mobilis pdc gene. This poor performance is due in large part to the accumulation of acetaldehyde, indicating a requirement for additional ADH activity. The level of expression of the Z. mobilis pdc and adhB genes is also an important consideration. Excessive levels of the Z. mobilis enzymes, such as observed with M5A1(pLOI297), appear to retard growth and slow fermentation.

The instability of high-copy-number plasmids with the colEl replicon in K. planticola (29) was also confirmed for M5A1. Neither the replicon nor the promoter expressing the Z. mobilis genes is known for pLOI555. However, this plasmid was maintained well and appears to provide near-optimal levels of PDC and ADH activities for ethanol production.

Expression of pdc in M5A1(pLOI560) resulted in an increase in the production of succinate. Succinate levels were not reported in analogous constructs of E. chrysanthemi (28) or K. planticola (29). No increase in succinate was observed in E. coli ED8767 (pZM11pdc) during anaerobic growth without pH control (3). Simultaneous expression of

TABLE 3. Ethanol production from glucose and xylose by recombinant strains of K. oxytoca M5A1

<table>
<thead>
<tr>
<th>Plasmid genes*</th>
<th>Base (mmol/g of sugar)</th>
<th>Time* (h)</th>
<th>Ethanol yield</th>
<th>Theoretical yield (%)</th>
<th>VP (g/liter)*</th>
<th>30-h ethanol amt (g/liter)</th>
<th>Cell yield (g/g of sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No plasmid</td>
<td>1.0</td>
<td>48</td>
<td>15</td>
<td>0.16</td>
<td>31</td>
<td>1.1</td>
<td>15</td>
</tr>
<tr>
<td>pLOI560 pdc</td>
<td>0.8</td>
<td>72</td>
<td>44</td>
<td>0.46</td>
<td>90</td>
<td>1.1</td>
<td>15</td>
</tr>
<tr>
<td>pLOI297 pdc adhB</td>
<td>0.7</td>
<td>72</td>
<td>50</td>
<td>0.52</td>
<td>102</td>
<td>1.3</td>
<td>24</td>
</tr>
<tr>
<td>pLOI555 pdc adhB</td>
<td>1.0</td>
<td>48</td>
<td>48</td>
<td>0.50</td>
<td>98</td>
<td>2.1</td>
<td>43</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No plasmid</td>
<td>1.9</td>
<td>96</td>
<td>14</td>
<td>0.16</td>
<td>31</td>
<td>0.5</td>
<td>7</td>
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<tr>
<td>pLOI560 pdc</td>
<td>0.7</td>
<td>96</td>
<td>37</td>
<td>0.38</td>
<td>75</td>
<td>1.2</td>
<td>5</td>
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<tr>
<td>pLOI297 pdc adhB</td>
<td>0.9</td>
<td>96</td>
<td>37</td>
<td>0.39</td>
<td>76</td>
<td>1.0</td>
<td>12</td>
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<tr>
<td>pLOI555 pdc adhB</td>
<td>1.1</td>
<td>48</td>
<td>46</td>
<td>0.48</td>
<td>94</td>
<td>2.0</td>
<td>37</td>
</tr>
</tbody>
</table>

* Calculations are based on total sugar added initially.
* Genes indicated are from Z. mobilis.
* Amount of base consumed to maintain a pH of 6.0 during fermentation.
* Time of maximum ethanol concentration.
* VP, Maximum volumetric productivity during batch fermentation.
adHB did not prevent the increase in succinate in the three recombinants of M5A1 expressing pdc. The level of succinate was similar in all three recombinants despite the expression of only one-eighth of the level of PDC in M5A1(pLOI555). Both pdc and adHB are assembled into an artificial operon (11), and similar differences are expected in the level of Z. mobilis ADHII. The lack of a large difference in succinate production between these M5A1 recombinants which expressed different levels of PDC is consistent with a lack of direct competition between the enzyme which commits glycolytic intermediates to succinate (phosphoenolpyruvate carboxylase) and PDC.

Four fermentation pathways function in *Klebsiella* strains (19): the pyruvate formate-lyase pathway, which produces formate (hydrogen and carbon dioxide) and acetate plus ethanol in equimolar amounts; the lactate acid pathway; the succinate pathway; and the butanediol pathway. All but the last of these also functions in *E. coli* (51). In both organisms, it has been possible to divert more than 90% of carbon flow from sugar catabolism away from the native fermentative pathways to ethanol. Partial success has been achieved with the pdc gene alone in *E. chrysanthemi* (28) and *K. planticola* (29). In these cases, the low $K_m$ of PDC for pyruvate allows this enzyme to compete effectively even in the presence of native pathways. Genetic approaches to metabolic engineering of other pathways have also been successful. The pathways for antibiotic biosynthesis have been merged in actinomycetes to produce novel pathways (9). Pathways encoding the synthesis of storage polymers (27) and carotenoids (17) have been functionally transplanted from one organism to another and have been integrated into the host metabolism. These results from different laboratories are representative of the interchangeable nature of metabolic pathways in microorganisms and the potential for metabolic engineering. As with the increased production of succinate, the integration of foreign pathways may have unexpected consequences and are not always predictable. However, nature has provided a marvelous diversity of metabolic activities in contemporary organisms. With a foundation of knowledge in microbial physiology and biochemistry and the tools of genetics, rational metabolic engineering of new recombinant organisms should provide an important route for the development of fine chemicals, chemical feedstocks, and fuels from contemporary biomass.

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Ethanologenic Klebsiella strain 2815


