Biosynthetic Burden and Plasmid Burden Limit Expression of Chromosomally Integrated Heterologous Genes (*pdc, adhB*) in *Escherichia coli*

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Previous studies have shown an unexpectedly high nutrient requirement for efficient ethanol production by ethanologenic recombinants of *Escherichia coli* B such as LY01 which contain chromosomally integrated *Zymomonas mobilis* genes (*pdc,adhB)* encoding the ethanol pathway. The basis for this requirement has been identified as a media-dependent effect on the expression of the *Z. mobilis* genes rather than a nutritional limitation. Ethanol production was substantially increased without additional nutrients simply by increasing the level of pyruvate decarboxylase activity. This was accomplished by adding a multicopy plasmid containing *pdc* alone (but not *adhB* alone) to strain LY01, and by adding multicopy plasmids which express *pdc* and *adhB* from strong promoters. New strong promoters were isolated from random fragments of *Z. mobilis* DNA and characterized but were not used to construct integrated biocatalysts. These promoters contained regions resembling recognition sites for 3 different *E. coli* sigma factors: σ^{70}, σ^{38}, and σ^{28}. The most effective plasmid-based promoters for fermentation were recognized by multiple sigma factors, expressed both *pdc* and *adhB* at high levels, and produced ethanol efficiently while allowing up to 80% reduction in complex nutrients as compared to LY01. The ability to utilize multiple sigma factors may be advantageous to maintain the high levels of PDC and ADH needed for efficient ethanol production throughout batch fermentation. From this work, we propose that the activation of biosynthetic genes in nutrient-poor media creates a biosynthetic burden that reduces the expression of chromosomal *pdc* and *adhB* by competing for transcriptional and translational machinery. This reduced expression can be viewed as analogous to the effect of plasmids (plasmid burden) on the expression of native chromosomal genes.

Introduction

Recombinant strains of *Escherichia coli* B were previously engineered for the bioconversion of hemicellulose-derived sugars into fuel ethanol by integrating the promoterless *Zymomonas mobilis pdc* and *adhB* genes encoding the ethanol pathway into the *pfl* gene (*14*). Criteria used to select *E. coli* B for metabolic engineering included the broad range of sugars fermented, environmental hardiness, and the ability to grow well in minimal medium containing only mineral salts (*1*, *24*). The *pfl* gene was selected for integration because it is constuitively expressed at high levels from up to 7 consecutive promoters (*29*). However, subsequent studies have identified many regulatory factors that modulate the level of *pfl* expression (*9*, *26*, *30*). Most fermentation studies reported with ethanologenic *E. coli* have been conducted with laboratory nutrients, although the development of inexpensive media is essential for commercial fuel ethanol production (*14*).

Our laboratory (*2*, *36*, *37*) and others (*18*) have recently investigated several inexpensive nutrients with unexpected results. Despite the prototrophic nature of the parent, the recombinant strains of *E. coli* B did not produce ethanol efficiently without the addition of large amounts of complex nutrients. For the complete fermentation of 90−100 g/L sugar to 40−50 g/L ethanol, at least 10 g/L crude yeast autolysate (*36*) or 10 g/L enzymatic hydrolysate of soy (*37*) was required in addition to mineral supplements. Alternatively, these nutrients could be replaced by the addition of 50 g/L corn steep liquor (50% solids) without added minerals (*2*). Such large requirements for complex nutrients are not readily explained when compared to the low level of biocatalyst present during fermentation (approximately 3 g/L cell dry weight).

In this study, we have investigated the basis of the nutrient requirement for ethanol production by ethanologenic *E. coli* B derivatives and discovered that it results from a media-dependent effect on the expression of the integrated *Z. mobilis* genes rather than a specific nutritional need.

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Table 1. Strains and Plasmids Used in This Study[a]

plasmid/strain	relevant genotype	reference
E. coli B	prototrophic	ATCC11303
E. coli DH5α	recA	Promega
E. coli LY01	*frd, pfl::pdc adhB cat*, alc^R	Yomano et al., 1998 (*35*)
pUC19	*bla*	Promega
pLOI276	*bla*, P_{lac} *pdc*	Conway et al., 1987 (*6*)
pLOI284	*bla*, P_{lac} *adhB*	Conway et al., 1987 (*7*)
pLOI308	*bla*, P_{neg} *pdc adhB*	Ingram and Conway, 1988 (*13*)
pLOI1591	*bla*, P_{Zm1} *pdc adhB*	this study
pLOI1592	*bla*, P_{Zm2} *pdc adhB*	this study
pLOI1593	*bla*, P_{Zm3} *pdc adhB*	this study
pLOI1594	*bla*, P_{Zm4} *pdc adhB*	this study
pLOI1595	*bla*, P_{Zm5} *pdc adhB*	this study

[a] Plasmid pLOI308 lacks a promoter for the expression of *pdc* and *adhB*. Plasmids pLOI1591−1595 contain fragments of *Z. mobilis* DNA. Abbreviations: P_{zm} refers to fragments of *Z. mobilis* DNA (pLOI1591−1595), which serve as strong promoters in *E. coli*; alc^R refers to mutation(s) in LYO1, which increase ethanol resistance.

Materials and Methods

Organisms and Stock Cultures. Strains and plasmids used in this study are listed in Table 1. Ethanologenic derivatives of *E coli* B were used as the biocatalysts for all fermentations. These organisms were genetically engineered to produce ethanol and CO_2 as the primary products of fermentation by the addition of *Zymomonas mobilis pdc* and *adhB* genes, which encode the pyruvate to ethanol pathway (*14*). Both genes are chromosomally integrated in LY01 (*25*,*35*). LY01 also contains a deletion in fumarate reductase, which blocks succinate production. Other ethanologenic *E. coli* B contained pUC-based plasmids expressing *Z. mobilis pdc* (pLOI276) (*6*), *adhB* (pLOI284) (*7*), or both (pLOI1591−1595). In some cases, unmodified *E. coli* B or LY01-(pUC19) was included as a control.

Stock cultures of LY01 were maintained on modified Luria-Bertani medium (*3*) that contained per liter: 5 g of Difco yeast extract, 10 g of Difco tryptone, 5 g of NaCl, 20 g of xylose, 15 g of agar, 10 g of 2-propanol, and 40 or 600 mg of chloramphenicol (*35*). Cultures were transferred daily on 2-propanol plates alternating between high and low chloramphenicol concentrations. Ethanologenic genes were tested using aldehyde indicator plates (*7*). Stock cultures of strains harboring plasmids were maintained on Luria-Bertani medium (LB) supplemented with 20 g/L xylose and 50 mg/L ampicillin. Frozen stocks were stored in 40% glycerol at −75 °C.

Media for Growth and Fermentation. Several different media were used in these experiments: LB supplemented with 50 g/L xylose, M9 (*3*) supplemented with 50 g/L xylose (adjusted to pH 7.0 with 2 N KOH), 10 g/L corn steep liquor mineral salts medium (1% CSL+M) containing 50 or 90 g/L xylose, and bagasse hemicellulose hydrolysate treated with lime (*2*) and supplemented with corn steep liquor (with and without mineral supplements) or LB nutrients (omitting NaCL). Lime-treated hemicellulose hydrolysate contained 83 g/L total sugar (primarily xylose) and was amended with xylose to provide a final sugar concentration of 100 g/L. Mineral supplements for 1% CSL+M medium and for hemicellulose hydrolysate (where indicated) were as follows (per liter): 1 g of KH_2PO_4, 0.5 g of K_2HPO_4, 3.1 g of $(NH_4)_2SO_4$, 0.4 g of $MgCl_2 \cdot 6H_2O$, and 20 mg of $FeCl_3 \cdot 6H_2O$. A stock solution of iron was prepared by dissolving 20 g of $FeCl_3 \cdot 6H_2O$ in 175 mL of HCl and adjusting to 1 L with sterile water. This iron stock also served as a source of trace metals (*37*). Xylose, Difco reagents, and mineral stocks were sterilized by autoclaving. Thiamine (M9) was sterilized by filtration. Corn steep liquor was adjusted to pH 7.2 with NaOH prior to autoclaving and aseptically clarified by centrifugation (10 min at 12000*g*) for growth studies and seed production. Neither corn steep liquor nor hydrolysate was sterilized (or clarified) prior to use in pH-controlled fermentations. M9 was prepared using deionized water, and all other media were prepared using tap water.

Nutritional Supplements. To evaluate the requirement for small molecules, we tested the following as amendments to 1% CSL+M media with xylose (50 g/L). Amendments were as follows per liter: 50 mg of each amino acid, 50 mg of each base (adenine, guanine, thymine, cytosine, and uracil), 200 mg of sodium acetate, or a mixture of vitamins (2 mL/L; ref *37*). Purines and pyrimidines were added as a combined stock. Amino acids were grouped into families for these tests: aromatic family (histidine, phenylalanine, tyrosine, and tryptophan); glycine family (glycine, serine, and cysteine); alanine family (alanine, lysine, diaminopimelic acid, valine, and leucine); aspartate family (methionine, aspartate, threonine, and isoleucine); and glutamate family (proline, glutamate, and arginine). Stocks were sterilized by autoclaving.

Inocula and Fermentations. Each strain was adapted by serial transfers using the corresponding solid medium (1.5% agar) containing 20 g/L xylose. Three fresh colonies of each strain were transferred to 13 × 100 mm culture tubes containing 3 mL of the appropriate medium and allowed to grow with agitation at 30 °C for 30 min (LB), 1 h (1% CSL+M), or 2 h (M9). Resulting suspensions were used to inoculate 500 mL flasks containing 150 mL of media (50 g/L xylose) to an initial OD_{550nm} of 0.005. Flasks were incubated for 16 h at 35 °C with agitation (120 rpm). Samples were removed for the measurement of OD, pH, ethanol, and enzymatic activities. Alternatively, these cultures were used as inocula (10%) for pH-controlled batch fermentations (pH 7.0, 35 °C, 100 rpm) as described previously (*4*).

ADH and PDC Activities. Alcohol dehydrogenase (ADH) activity was estimated by measuring ethanol oxidation at pH 8.5 as described previously (*20*, *23*). Pyruvate decarboxylase (PDC) activity was assayed by monitoring the pyruvic acid-dependent reduction of NAD+ with alcohol dehydrogenase as a coupling enzyme at pH 6.5 (*6*, *20*) using the method of Hoppner and Doelle (*12*). Activities are reported as IU per milligram of cell protein. Cell protein was estimated using a standard curve of protein concentration versus OD_{550nm} (1.0 OD_{550nm} corresponds to 0.23 mg of cell protein per milliliter; ref *20*).

Genetic Methods. Standard procedures were used for the construction, isolation, and analysis of plasmids (*27*). Chromosomal DNA was isolated previously from *Z. mobilis* CP4 (*34*) and was digested with *Sau*3A1 to provide a source of surrogate promoters. Dideoxy sequencing of promoter fragments was performed by using fluorescent primers and a LI−COR model 4000L DNA sequencer as previously described (*17*). Sequences were analyzed using the Wisconsin Version 9.1, Genetics Computer Group (GCG), software package (*8*) and the National Center for Biotechnology Information BLAST network service (*5*). The nucleotide sequences of the surrogate promoters in plasmids pLOI1591−pLOI1595 have been assigned GenBank accession numbers AF105011, AF105012, AF105013, AF105014, and AF105015, respectively.

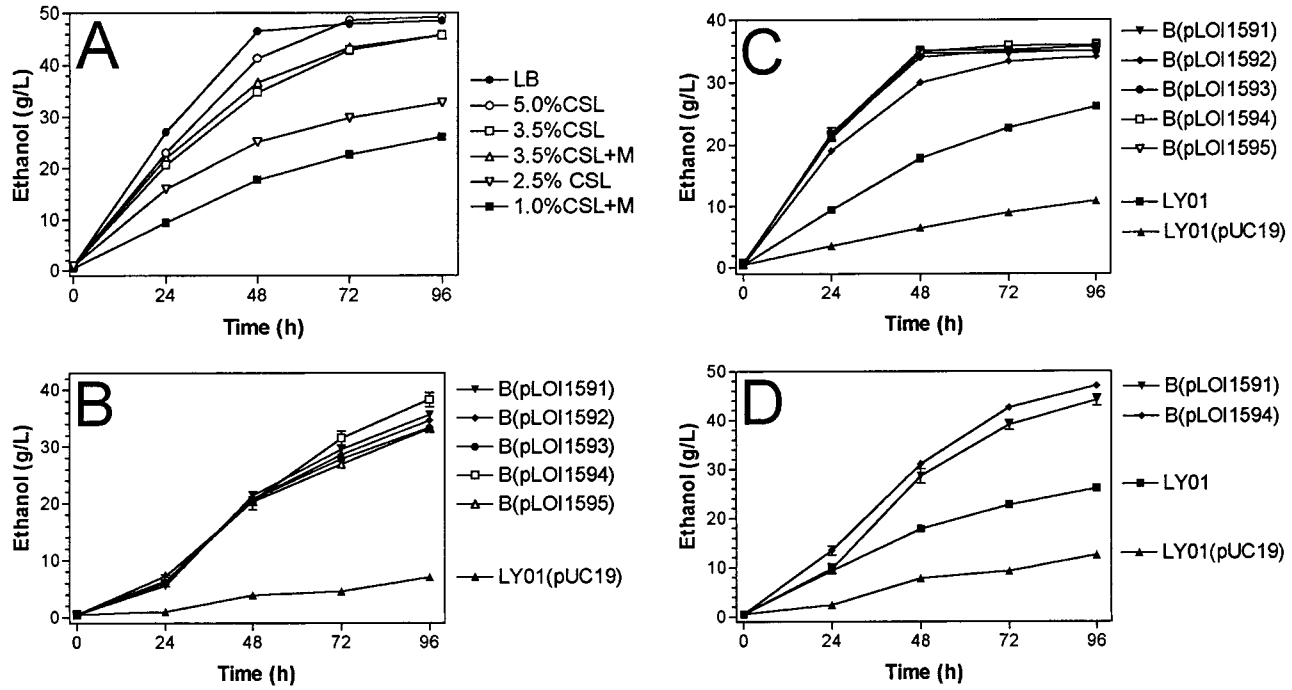

Figure 1. Fermentation of sugars by ethanologenic *E. coli* in pH-controlled fermentors. (A) Bagasse hemicellulose hydrolysate containing LB nutrients (100 g/L total sugar) and LY01 as the biocatalyst. Hydrolysate was amended with corn steep liquor and mineral salts as indicated. (B) Comparison of different strains fermenting 1% CSL+M (90 g/L xylose). (C) Comparison of different strains fermenting hydrolysate containing 1% corn steep liquor plus minerals (90 g/L total sugar). (D) Comparison of different strains fermenting bagasse hydrolysate containing 2% corn steep liquor plus minerals (100 g/L total sugar).

Primer Extension Analysis of Promoters. Promoter regions were identified by mapping transcriptional start sites using a primer within the *pdc* gene. RNA was isolated from cells in late exponential phase using a Qiagen RNeasy kit. Prior to lysis, cells were treated with 400 μg/mL lysozyme in TE plus 0.2 M sucrose at 25 °C for 5 min. RNA was dissolved in 20 μL of Promega AMV reverse transcriptase buffer. IRD41-labeled primer (ctaaagtgtcgttccggatttagctatagg, LI−COR Inc.) was added and the mixture denatured for 5 min at 80 °C, annealed for 1 h at 55 °C, and purified by alcohol precipitation. For extension, samples were dissolved in 19 μL of AMV reverse transcriptase buffer containing 500 μM dNTPs and 10 units/μL AMV reverse transcriptase and incubated for 1 h at 42 °C. Products were treated with 0.5 μg/mL DNase-free RNase A, alcohol precipitated, dissolved in loading buffer, and compared to parallel dideoxy sequences using the LI−COR model 4000L DNA sequencer.

Analytical Procedures. Cell mass was estimated by measuring OD_{550nm} using a Baush & Lomb Spectronic 70 spectrophotometer. Ethanol was measured by gas chromatography using a Varian 3400 CX gas chromatograph with 1-propanol as an internal standard (*4*).

Results and Discussion

Comparison of LY01 Growth and Fermentation in Different Media. As shown in Figure 1A, strain LY01 requires at least 50 g/L of corn steep liquor to support ethanol production rates equivalent to 15 g/L of Difco nutrients (LB) in pH-controlled fermentors containing hemicellulose hydrolysate. Lower rates of fermentation and lower ethanol concentrations were observed upon reduction of corn steep liquor to 35 g/L. Reduction of corn steep liquor concentrations from 50 g/L to 35 and 10 g/L resulted in a drastic decline in yield after 96 h from 94% to 65% and 51%, respectively. Comparisons in flask cultures without pH control (50 g/L xylose) confirmed this large nutrient requirement (Figure 2A). After 16 h of incubation, over 10 g/L ethanol was produced in LB medium, while less than 2.5 g/L ethanol was produced in 1% CSL+M medium and less than 1 g/L ethanol was produced in M9 medium. The ratio of ethanol/cell mass (OD_{550nm}) was above 1 in LB, below 1 in 1% CSL+M, and less than 0.2 in M9 medium, indicating that growth of the biocatalyst alone is insufficient to account for the large variation in ethanol production.

CSL is known to be a good source of vitamins and small molecules. However, the poor growth and ethanol production by LY01 in 1% CSL+M medium (Figure 2A) and the decrease in performance in pH-controlled fermentors with 35 g/L versus 50 g/L corn steep liquor (Figure 1A) suggested the possibility of a limiting nutrient. Three hypotheses were investigated: (1) insufficient mineral macro nutrients or trace metals; (2) an auxotrophic requirement which develops during ethanol production; (3) an acetate requirement resulting from disruption of the *pfl* gene during chromosomal integration. The addition of iron and mineral nutrients did not improve the fermentation of hydrolysate containing 35 g/L corn steep liquor (Figure 1A). To examine potential auxotrophic requirements, we compared the growth of LY01 to the unmodified parent, *E. coli* B (Figure 2A). In all media, cell densities after 16 h for LY01 exceeded those of *E coli* B. To further examine a potential auxotrophic requirement, a mixture of vitamins, a mixture of purines and pyrimidines, and families of amino acids were added to 1% CSL+M medium. Each addition resulted in only a small increase in ethanol production and growth (Figure 3) when compared to the 3-fold higher cell mass and ethanol with LB (Figure 2A). Surprisingly, the final pH values of cultures grown in 1% CSL+M (with and without supplements) were essentially the same as that of LB, pH 5.

Pyruvate formate lyase is a primary route for the production of acetyl-CoA (lipid biosynthesis) during

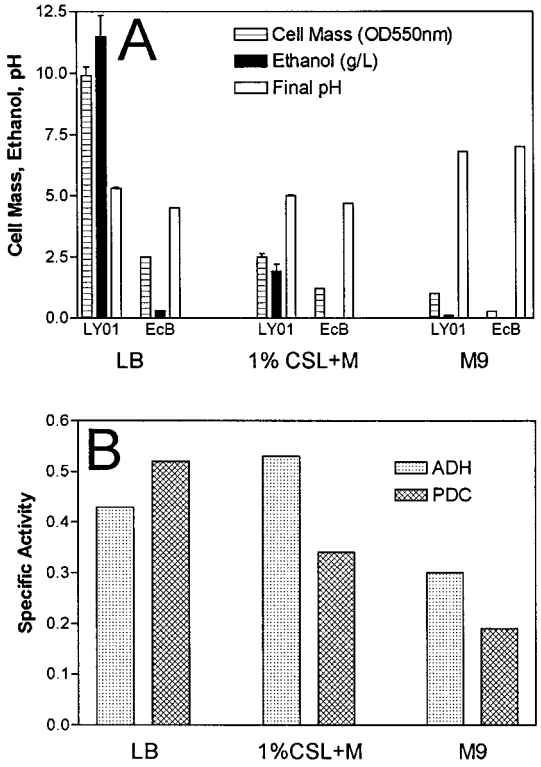

Figure 2. Effect of growth media on ethanol production and recombinant enzyme activities in LY01. All media contained 50 g/L xylose. Flask cultures were incubated for 16 h at 35 °C. (A) Comparison of final pH, cell mass, and ethanol production by LY01 and *E. coli* B. (B) Comparison of PDC and ADH activities (IU/mg protein) in strain LY01.

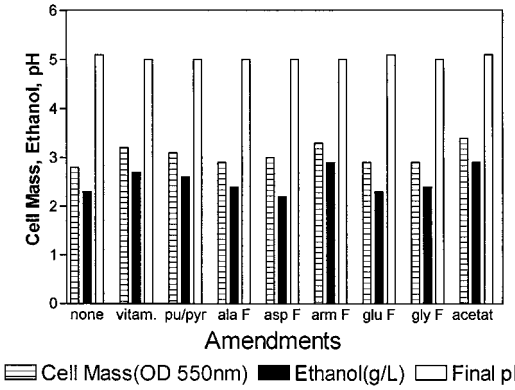

Figure 3. Effects of nutritional amendments to 1% CSL+M medium (50 g/L xylose) on growth and ethanol production by LY01. Flask cultures were incubated for 16 h at 35 °C. Abbreviations: vitam., vitamin mix; pu/pyr, mixture of purines and pyrimidines; ala F, alanine family of amino acids; asp F, aspartic acid family of amino acids; arm F, aromatic family of amino acids; glu F, glutamic acid family of amino acids; gly F, glycine family of amino acids; acetat, sodium acetate.

anaerobic growth (*11*). In some strains of *E. coli*, disruption of *pfl* leads to acetate auxotrophy which could limit growth and fermentation. However, addition of 200 mg/L sodium acetate to 1% CSL+M medium resulted in only a small increase in growth and ethanol production (Figure 3), similar to that observed with the addition of aromatic amino acids.

Together, these results demonstrate that growth and ethanol production are not limited by minerals or specific auxotrophic requirements. All organic nutrients tested provided only a small incremental benefit.

Expression of Integrated Heterologous Genes in Different Media. The similarity in final pH among

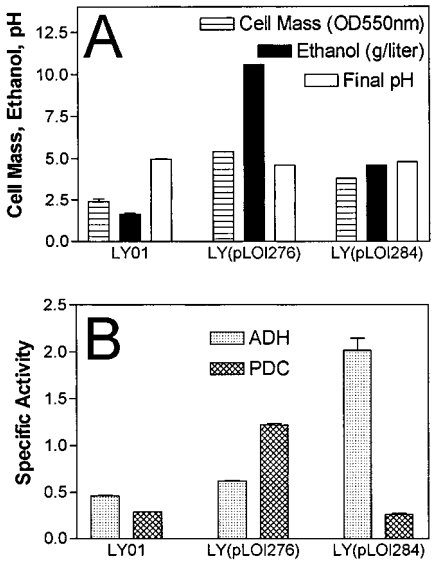

Figure 4. Effects of plasmids expressing *Z. mobilis pdc* and *adhB* on growth and ethanol production by LY01 and *E. coli* B derivatives in 1% CSL+M medium (50 g/L xylose). Flask cultures were incubated for 16 h at 35 °C. (A) Comparison of final pH, cell mass, and ethanol. (B) Comparison of PDC and ADH activities.

cultures grown in 1% CSL+M and LB suggests that acidification may limit growth and ethanol production in shaken flasks (Figures 2A and 3). Since previous studies have shown that high levels of PDC and ADH are required to effectively divert metabolism to ethanol and prevent lactic acid production (*13*), we have examined the levels of these enzymes in cells grown in different media (Figure 2B). PDC activity was found to directly correlate with the nutritional completeness of the media. The highest activity was present in cells grown in LB, while the lowest activity was found in cells grown in M9 medium. Thus, the nutrient levels in the growth media appear to directly or indirectly regulate *pdc* expression.

To test the hypothesis that the apparent high nutrient requirement for ethanol production by LY01 directly results from inadequate expression of *Z. mobilis* genes, we have inserted pUC-based plasmids containing either *pdc* (pLOI276) alone or *adhB* (pLOI284) alone and compared growth and ethanol production in different media (Figure 4A). As expected, addition of these plasmids increased the specific activities of PDC and ADH, respectively (Figure 4B). Insertion of plasmid pLOI276 containing *pdc* restored ethanol production in 1% CSL+M medium to a level equivalent to that for LY01 in LB medium. Although cell growth in these plasmid-containing recombinants was lower than that of unmodified LY01 in LB, the LY01(pLOI276) containing *pdc* produced a higher ratio of ethanol/cell mass in 1% CSL+M medium than unmodified LY01 grown in LB. The addition of plasmid pLOI284 containing *adhB* caused a smaller increase in growth and ethanol production, indicating an additional need for higher levels of ADH.

Effect of Plasmid Burden on the Expression of Integrated Heterolgous Genes. High copy number plasmids frequently slow the growth of recombinant strains by competing with cellular biosynthetic processes, termed plasmid burden (*21*, *32*). An analogous negative effect on the expression of integrated heterologous genes was observed with flask cultures containing 1% CSL+M medium. The addition of pUC19 to LY01 dramatically reduced cell growth, ethanol production, and the specific

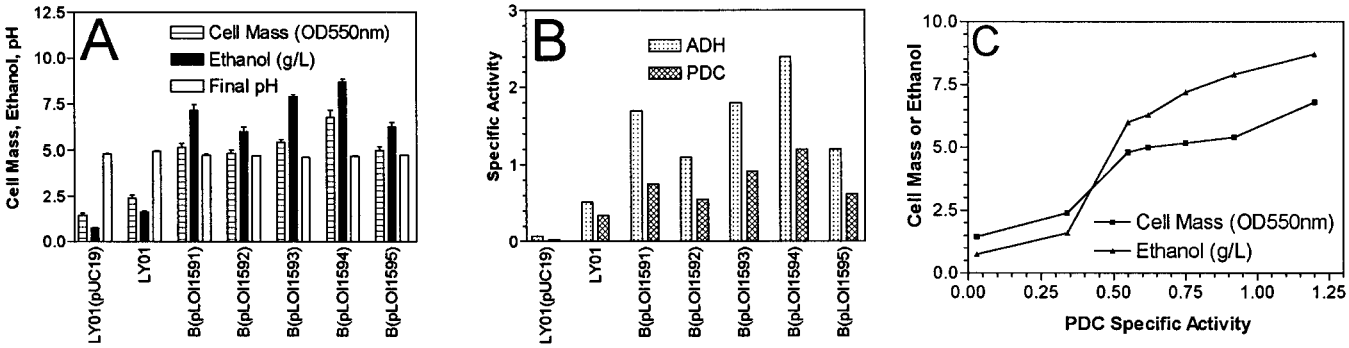

Figure 5. Fermentation by *E. coli* B derivatives harboring plasmids expressing *Z. mobilis pdc* and *adhB*. Flask cultures containing 1% CSL+M (50 g/L xylose) were incubated for 16 h at 35 °C. (A) Comparison of growth and ethanol production. (B) Comparison of PDC and ADH activities (IU/mg protein). (C) Combined data for ethanol production and PDC activity from all recombinant strains.

activities of PDC and ADH (Figure 5A,B). The plasmid burden of this high copy plasmid was also evident during batch fermentations using CSL-based media (Figure 1B,D). After 96 h, the ethanol yield for LY01(pUC19) was less than half that of unmodified LY01 with laboratory sugars and with hemicellulose hydrolysate as the primary substrate.

Construction of Plasmid-Based Ethanologenic Biocatalysts with New Promoters. The *pfl* gene was used as the *E. coli* integration site for the promoterless *Z. mobilis pdc*, *adhB* in LY01 (*35*). In *E. coli*, *pfl* is expressed from multiple, consecutive promoters (*29*). Although this promoter was adequate during growth in LB, our results indicate that this promoter does not provide sufficient expression of *pdc* and *adhB* during growth in M9 or 1% CSL+M media. A simple approach to further verify this hypothesis is to make recombinants containing multi-copy plasmids with both ethanol pathway genes, comparing *pdc* and *adhB* expression to performance in batch fermentation. The *lac* promoter used in pLOI276 and pLOI284 was not used due its regulation by sugars.

New plasmids were constructed with surrogate promoters from *Z. mobilis* chromosomal DNA to minimize the opportunity for regulation. *Sau*3A1 fragments were randomly inserted into the unique *Bam*H1 site of pLOI308, which contains a promoterless *pdc*,*adh*B cassette. Ligation products were transformed into *E. coli* B and directly screened for ADH activity on aldehyde indicator plates (*7*). Constructs which grew well on M9 plates and produced bright red colonies on indicator plates were purified and retested. After many rounds of comparison, plasmid DNAs were isolated from the five clones that appeared most active and transformed into a *recA* host (DH5∝) for maintenance.

Plasmid DNA isolated from DH5∝ recombinants was transformed back into *E. coli* B to confirm the phenotype. The resulting *E. coli* B constructs (designated pLOI1591−pLOI1595) were maintained on M9 minimal medium containing ampicillin and xylose.

The surrogate promoters from *Z. mobilis* were sequenced in both directions, and all were unique. Sizes ranged from 74 base pairs for pLOI1592 to 475 base pairs for pLOI1594. All were AT-rich and contained multiple regions resembling enteric promoters. No strong matches were found in the current DNA databases. Comparison of translated sequences (all reading frames) revealed only one strong match. The translated sequence in pLOI1595 was similar to the middle portion of an *E. coli* cell division protein (*ftsH*). Weak matches to other *E. coli* proteins were also observed for pLOI1593 (cysteinyl-tRNA synthetase; *cysS*) and pLOI1594 (UDP-*N*-acetylglucosamine carboxyvinyl transferase, *murZ*).

Transcriptional start sites were identified by primer extension analysis (Table 2). All except pLOI1591 contained two consecutive start sites, indicating the presence of at least 2 promoters. A comparison of upstream regions revealed the presence of sequences that are similar to the motifs utilized by the three most abundant *E. coli* sigma factors (*10*, *16*, *33*): σ^{70} (*rpoD*), σ^{38} (*rpoS*), and σ^{28}

Table 2. Putative Promoter Regions for *Z. mobilis* DNA Fragments Which Serve as Surrogate Promoters[a]

Plasmid	−35 region	−10 region	Start	Proposed sigma factor(s)
pLOI1591	cgactctagagGATCAGGAAAAGATATGGCATGAT	AAAGC	G	σ^{28}
pLOI1592	GATCGGAAGCCAGTCTTGCAAGCGGCGTATTGT	GAACACC	A	σ^{38}
	CCACATTCCAAAATATCTAATTTTGGGGGCTATAAT	AATAC	G	σ^{70}, σ^{28} GGGCTATA
pLOI1593	AAGCGGGTATCCATCCTTTGGCTTATCGGGTATT	ATGCTT	G	σ^{38}
	AGAAAAAGGTGGTTGCTGACGGTGAACCGGATT	GGTTGCG	G	σ^{38}, σ^{28} CCGGATTG
pLOI1594	GATATTGTGAAGCGGTATCAGCTTAAAAATAT	CGGATTTG	A	σ^{70}
	CGGATTTGAGAAGTTAAATTCGGCATGGCATACT	GATGCC	G	σ^{70}, σ^{38} CGGATT
pLOI1595	TGCCTCTTGTGACTGGGCGCATGAACGCTTTACT	ATGGAT	G	σ^{70}, σ^{38} CCTCTT
	AAGCCGTTATCAAAGTTTTCGTTTCGCTTTATAAT	GAAGG	C	σ^{70}, σ^{38} GCCGTT

[a] Transcriptional starts for *pdc* operon fusions were identified by primer extension analysis. Upstream sequences related to *E. coli* −35 regions and −10 regions are marked with double and single underlines, respectively. Two promoters were identified in pLOI1592, pLOI1593, pLOI1594, and pLOI1595. The lower promoter (bold start base) was dominant. For some promoters, two potential transcription factors were identified. Alternative recognition sequences for the second factor are listed on the right. *Z. mobilis* sequence is shown in upper case. Vector sequence is shown in lower case.

(*rpoF*). In all except pLOI1591, it is possible that the same promoter regions may function with multiple sigma factors.

Growth, Ethanol Production, and Gene Expression of New Recombinants in Flask Culture. Figure 5, parts A and B, show a comparison of the five new constructs and LY01 grown in 1% CSL+M medium. Growth, ethanol production, and expression of both *Z. mobilis* genes by all plasmid constructs were 2−3-fold higher than LY01 (Figure 5A,B). Both growth and ethanol production by these strains were directly related to the specific activities of PDC and ADH (Figure 5C). Growth of *E. coli* is typically limited by acidification of the media (*13*). At low PDC activities, cell mass increases rapidly with PDC due to the reduction of acidic products. However, high levels of PDC compete with biosynthesis for pyruvate and reducing energy, limiting cell growth, and producing higher ratios of ethanol to cell mass.

The highest levels of PDC activity and ethanol production were produced by pLOI1594, which appears to contain two σ^{70} and one σ^{38} promoter regions. Plasmids pLOI1593 and pLOI1591 were almost as effective and appear to lack the σ^{70} promoter region. On the basis of primer extension and upstream sequence, these two plasmids are presumed to express the *pdc* and *adhB* genes using σ^{38} and σ^{28}. Sigma factors have been shown to vary in abundance during the growth cycle (*16*). During batch fermentations, the ability to utilize multiple sigma factors may prove advantageous to maintain the high levels of PDC and ADHII needed for efficient ethanol production.

Nutrient requirements were reduced in flask fermentation by increasing the expression of *pdc* and *adhB* genes that encode the ethanol pathway. This was accomplished by the addition of multicopy plasmids containing both genes to *E. coli* B or supplementing expression from the integrated genes by adding *pdc* alone. In flask cultures without pH control, however, over half of the sugar remained unmetabolized. Further comparisons were made using pH-controlled fermentors (Figure 1B,C,D). None of the strains fermented well in M9 medium containing 90 g/L xylose (data not included). However, all new strains produced ethanol at a higher rate than LY01 in 1% CSL+M containing 90 g/L xylose (Figure 1B) and in hydrolysate supplemented with either 10 or 20 g/L corn steep liquor plus minerals (Figure 1, parts C and D, respectively). After 96 h, ethanol concentrations produced by two of the new constructs (pLOI1591 and pLOI1594) with 20 g/L corn steep liquor were equivalent to LY01 with 50 g/L corn steep liquor and LY01 with LB. With hydrolysate containing 1% CSL+M, all five constructs produced ethanol at least 3-fold faster than LY01(pUC19). However, the initial rates of ethanol production with LB remained substantially faster than with corn steep liquor supplemented fermentations.

Increasing gene expression through the replacement of promoters and the use of a higher gene dosage (plasmids) substantially eliminated the apparent requirement for large amounts of complex nutrients in shaken flasks and pH-controlled fermentors. These results further confirm that inadequate expression of *pdc* and *adhB* rather than a nutritional requirement limit the performance of LY01 in the absence of abundant complex nutrients.

Biosynthetic Burden: a Unifying Hypothesis. The diversion of biosynthetic resources from native functions to the replication of plasmids and the expression of plasmid-borne genes typically results in a decrease in growth rate and vigor of the recombinant, that is, plasmid burden (*19*, *21*, *28*, *32*). In *Z. mobilis*, for example, overexpression of a single recombinant gene reduced the specific activities of chromosomally encoded glycolytic enzymes, resulting in slower glycolysis (*31*). The converse of this, biosynthetic burden, appears to occur in LY01 during a shift from LB to 1% CSL+M or M9 media. *Z. mobilis pdc* and *adhB* genes are chromosomally integrated in LY01 and are expressed at adequate levels for ethanol production during growth in LB. Upon shifting to media with less nutrients, repressed chromosomal genes would be activated for biosynthetic pathways. Activation of these additional genes appears to reduce *pdc* and *adhB* expression by out-competing the *pfl* promoter (controls expression of the integrated *Z. mobilis* genes) for the available transcriptional and translational machinery. Supplementation of 1% CSL+M medium with families of amino acids, purines and pyrimidines, and vitamins resulted in incremental improvements in ethanol production. Additional copies (plasmid-borne) of the *pdc* gene (*lac* promoter) increased ethanol production by LY01 in 1% CSL+M to near that observed in LB. In flask fermentations, the new biocatalysts containing promoters selected for high expression of *adhB* (and upstream *pdc*) in M9 medium produced as much ethanol in 1% CSL+M media as in LB. Finally, the addition of pUC19 to LY01, a plasmid burden which also competes for the biosynthetic machinery, reduced expression of chromosomal *pdc* and *adhB* by over half with a corresponding reduction in growth and ethanol production. Assuming that 200−500 copies of pUC19 are present per cell and that all plasmid genes are active, addition of pUC19 could be considered roughly equivalent to activating the transcription of an additional 30% of the *E. coli* chromosome.

E. coli has been extensively studied as a model for gene regulation and contains elaborate controls for the transcription and translation of genes comprising individual biosynthetic pathways. The activation of a full set of biosynthetic genes to synthesize all building block molecules and vitamins would be expected to compete with and reduce the expression of other genes whether chromosomal, plasmid-borne, native, or heterologous. Indeed, it is likely that changes in biosynthetic burden are in part responsible for media-dependent changes in the expression of many recombinant biotechnology products. An analogous competition for metabolic precursors, transcriptional machinery, and translational machinery rather than a limitation of energy (ATP) has been proposed to regulate the growth rate of *E. coli* in different media (*15*, *22*).

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