Engineering a Homo-Ethanol Pathway in *Escherichia coli*: Increased Glycolytic Flux and Levels of Expression of Glycolytic Genes during Xylose Fermentation†

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Replacement of the native fermentation pathway in *Escherichia coli* B with a homo-ethanol pathway from *Zymomonas mobilis* (*pdc* and *adhB* genes) resulted in a 30 to 50% increase in growth rate and glycolytic flux during the anaerobic fermentation of xylose. Gene array analysis was used as a tool to investigate differences in expression levels for the 30 genes involved in xylose catabolism in the parent (strain B) and the engineered strain (KO11). Of the 4,290 total open reading frames, only 8% were expressed at a significantly higher level in KO11 (*P* < 0.05). In contrast, over half of the 30 genes involved in the catabolism of xylose to pyruvate were expressed at 1.5-fold to 8-fold-higher levels in KO11. For 14 of the 30 genes, higher expression was statistically significant at the 95% confidence level (xyLA, xyLE, xyLF, xyIR, rpiA, rpiB, pfkA, fbaA, tpiA, gapA, pgk, and pykA) during active fermentation (6, 12, and 24 h). Values at single time points for only four of these genes (eno, fbaA, fbaB, and talA) were higher in strain B than in KO11. The relationship between changes in mRNA (cDNA) levels and changes in specific activities was verified for two genes (xyLA and *xyLB*) with good agreement. In KO11, expression levels and activities were threefold higher than in strain B for xylose isomerase (xyLA) and twofold higher for xylulokinase (*xyLB*). Increased expression of genes involved in xylose catabolism is proposed as the basis for the increase in growth rate and glycolytic flux in ethanologenic KO11.

Completion of the *Escherichia coli* genome sequence (4) and the development of gene array technology (5, 13) offers new approaches for investigating complex problems which affect the expression of many genes. Previous studies have reported differences in expression during the aerobic growth of *E. coli* on different carbon sources (24, 36), in minimal and complex media (36), and in response to heat shock (27). Additional studies have used isogenic strains to investigate the consequences of specific mutations in regulatory genes, *ihf* (1) and *marA* (2). Comparisons during anaerobic and aerobic growth of *Bacillus subtilis* (41) and *Saccharomyces cerevisiae* (37) have identified changes in more than 200 genes each. A variation of this technology has recently been developed by Khodursky et al. (16) with sufficient accuracy to monitor the movement of replication forks around the *E. coli* chromosome.

Expression arrays offer a unique opportunity to investigate the consequences of metabolic engineering and may provide clues to limitations in metabolic flux (24). The anaerobic fermentation of *E. coli* (pH controlled) is a particularly attractive system for such studies due to the simplicity of culture conditions and the lack of complications from changes in oxygen availability or pH. Our laboratory previously developed ethanologenic derivatives of *E. coli* B in which pyruvate metabolism was redirected to ethanol and carbon dioxide by the integration and functional expression of *Zymomonas mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) (14, 15, 25). A deletion was also introduced into the fumarate reductase gene (*frd*) to minimize succinate production. The resulting strain, KO11, efficiently ferments xylose at high concentrations of pentoses and hexoses. Surprisingly, KO11 appears to grow more rapidly than the parent on plates and in broth (L. O. Ingram, unpublished observations).

In this study, we report that both glycolytic flux and specific growth rate are higher in the ethanologenic strain KO11. The physiological basis for the increase in glycolytic flux was examined by comparing expression levels for the 30 genes associated with xylose catabolism to pyruvate using gene arrays, many of which were higher in KO11 (*P* < 0.05) than in strain B.

**MATERIALS AND METHODS**

**Strains and media.** *E. coli* B (ATCC 11303) and an ethanologenic derivative, KO11 (*Δfrd pff:pdcZm adhBZm cat*) (14, 25), were used in this study. Both strains are prototrophic. Cultures were stored at −75°C in 40% glycerol. Working stocks were maintained under argon on solid Luria-Bertani medium (LB) (per liter: 5 g of Difco yeast extract, 10 g of Difco tryptone, and 5 g of NaCl) containing 2% xylose and 1.5% agar (29). Chloramphenicol (0.6 mg/ml) was included in plates used to maintain working stocks of KO11. No antibiotics were added to seed cultures or fermentation broth.

**Fermentation.** Batch fermentations were conducted at 35°C in 500-ml stirred vessels (100 rpm) containing 350 ml of LB plus xylose (10%) as described previously (3). Fermentations were maintained at pH 6.0 by the automatic addition of KOH (2 N for KO11; 2 N for 0 to 24 h and 6 N for 24 to 96 h for strain B). Seed cultures were prepared by transferring fresh colonies from solid media into 2-liter flasks containing 720 ml of LB (5% xylose) and incubating for 10 h at 35°C (120 rpm). To inoculate fermentations, sufficient cell mass was harvested by centrifugation (5,000 × g, 5 min, 22°C) to provide an initial cell density of 0.33 g (dry weight) per liter (10% of maximum cell density). These inocula were trans-
ferred using a small amount of broth from each vessel. During fermentation, samples were removed for the measurement of optical density at 550 nm (OD550). pH, ethanol, xylose, and organic acids. Fermentation results are averages from four experiments for KO11 and two experiments for strain B.

Analysis of glucose fermentation. Five sets of UV-cross-linked arrays of 4,290 ORF-specific DNAs (10 ng per array) was separated from small molecules by gel filtration using a 1-ml Sephadex G50 column and resulted in approximately 70% incorporation of label. Radioactive cDNA was prepared by dividing volumetric rates by the concentration of cell protein (milligrams milliliters−1) and then calculating the specific volume of the cell protein (milligrams milliliters−1). Glycylclic flux was also calculated based on the production of fermentation products. The two methods were equivalent due to the small amount of carbon which is used for cell growth. The values from the 46- and 72-hour samples were not included in these calculations for KO11 due to the decline in cell mass and possible lysis resulting from xylose exhaustion.

Carbon balance. Total carbon recovery was excellent, with near closure at all points during fermentation. Carbon recoveries ranged from 96.2 to 101% for KO11 and 87.4 to 103% for strain B. Carbon dioxide was estimated as the molar sum of acetic acid and ethanol. The carbon content in biomass was assumed to be 48%.

Isolation of total RNA. Total RNA was isolated from E. coli cultures by a modified hot-phenol extraction method (36). A sample of the E. coli culture (0.2 to 1 ml; not exceeding 106 cells) was transferred to a tube containing 4 ml of hot lysis buffer (1% sodium dodecyl sulfate, 30 mM sodium acetate, 3 mM EDTA; pH 5.0) in a boiling water bath and held for 5 min with intermittent mixing. Resulting lysates were extracted twice with equal volumes of hot phenol (pH 5.0, 65°C), and once each with phenol-chloroform and then chloroform alone. Total RNA was precipitated with 2.5 volumes of 95% ethanol. The RNA pellet was washed twice with 70% ethanol, dried, and dissolved in 0.1 ml of sterile water. Contaminating DNA was removed from RNA by hydrolysis with DNase I (200 U, 37°C for 30 min; Life Technologies) and by column purification (Qiagen RNeasy Mini Kit). RNA concentration was determined from the absorbance at 260 nm.

Synthesis of radiolabeled cDNA. A set of primers homologous to the 3’ ends of the predicted 4,290 open reading frame (ORF) in E. coli was obtained from Sigma-Genosys (Panorama E. coli cDNA labeling primers). The average melting temperature (Tm) for the primers in this mixture is 66.7 ± 4.7°C. These primers were used to prepare cDNA based on a modification of the protocol provided by the manufacturer. Total E. coli RNA (1 µg), primer mix (4 µl), deoxynucleoside triphosphate mix (lacking dCTP) (0.33 mM each) and reverse transcriptase (200 U; Life Technologies), and appropriate units (milligrams of cell protein minute−1) by evaluating the first derivative at each sample time. After conversion to appropriate units (milligrams of cell protein minute−1), these values were normalized to the concentration of cell protein (milligrams milliliters−1). Glycylclic flux was also calculated based on the production of fermentation products. The two methods were equivalent due to the small amount of carbon which is used for cell growth. The values from the 46- and 72-hour samples were not included in these calculations for KO11 due to the decline in cell mass and possible lysis resulting from xylose exhaustion.

Analytical methods. Fermentation of xylose by E. coli. Figure 1 summarizes the pathway for the catabolism of xylose to pyruvate in E. coli and the fermentation pathway for KO11. Ethanol (and CO2) is the dominant fermentation product in KO11 (Fig. 2A). The parent organism, strain B, produces a mixture of acidic fermentation products (lactic acid, acetic acid, and succinic acid) and a small

RESULTS

Fermentation of xylose by E. coli. Figure 1 summarizes the pathway for the catabolism of xylose to pyruvate in E. coli and the fermentation pathway for KO11. Ethanol (and CO2) is the dominant fermentation product in KO11 (Fig. 2A). The parent organism, strain B, produces a mixture of acidic fermentation products (lactic acid, acetic acid, and succinic acid) and a small
amount of ethanol (Fig. 2B). As seen from a comparison of Fig. 2A and B, KO11 grew and catabolized xylose more rapidly than strain B. KO11 completed xylose catabolism within 48 h, while only 60% was catabolized by strain B after 96 h. The average rate of sugar utilization during fermentation for KO11 (2 g liter\(^{-1}\) h\(^{-1}\)) was over three times that for strain B (0.6 g liter\(^{-1}\) h\(^{-1}\)).

Glycolytic flux and specific growth rate were calculated at each time point as the first derivative of equations based on data for growth and fermentation (Fig. 3A and B). Shapes of the resulting curves for growth rate and flux were similar for both organisms, displaced only by magnitude and incomplete sugar utilization by strain B. Growth rate and flux were highest at the earliest time point (6 h). The maximum growth rate for

FIG. 1. Metabolism of xylose to pyruvate. Values for ATP correspond to the uptake and conversion of six molecules of xylose into 10 molecules each of ethanol and CO\(_2\).

FIG. 2. Fermentation of xylose (10%) by strain KO11 (ethanologenic) (A) and strain B (parent) (B). Standard deviations for four experiments are represented by error bars (panel A only). In some cases, error bars are hidden by symbols. Values in panel B are averages for two experiments.

FIG. 3. Comparison of specific growth rate (A), glycolytic flux (B), and expression of selected genes during batch fermentation correlated with glycolytic flux (C). Gene expression is reported as the percentage of bound cDNA (10\(^6\)). Standard deviations for each gene are shown in Fig. 5. To illustrate significance, dashed lines denoting the 95% confidence interval are included for eno.
KO11 was estimated as 0.224 h\(^{-1}\) at 6 h, approximately 1.3-fold that for strain B. Glycolytic flux remained higher in KO11 than in strain B until xylose was exhausted by KO11 (48 h). The maximum flux for KO11 was 0.278 \(\mu\)mol xylose min\(^{-1}\) mg of cell protein\(^{-1}\), approximately 1.5-fold higher than that of strain B at 6 h. At 12 h and 24 h, glycolytic flux was 1.7-fold higher in strain KO11 than strain B.

**cDNA measurements.** We used mRNA profiles to examine the hypothesis that the higher rate of glycolytic flux in KO11 than in strain B results from an increase in the expression of one or more of the 30 genes involved in xylose catabolism. Since considerable error exists in these measurements, with an average coefficient of variation (standard deviation expressed as a percentage of the mean) of 38%, \(P\) values were computed for each gene at each time point using a one-tailed Student \(t\) test.

Many steps are involved in estimating relative gene expression using gene arrays contributing to scatter in the results. Among the 4,290 ORFs, 210 measuring below 0.001% of the total bound cDNA had high standard deviations, often exceeding 50% of the measured value, and were judged unreliable. These ORFs were eliminated from further consideration. The coefficient of variation (standard deviation expressed as a percentage of the mean) was used to estimate the error associated with the measurement of the remaining ORFs. This coefficient averaged from 5 to 12% for the comparisons of ORFs measured on the duplicate arrays from a single filter and from 30 to 43% for multiple filters probed with cDNA from the same sample. During the active stages of growth and fermentation (6, 12, and 24 h), 8% of the 4,080 ORFs were expressed at significantly higher levels (\(P < 0.05\)) in KO11 than in strain B and an equal number were at lower levels. Larger differences were observed for later time points. However, results for these later times (36, 48, and 72 h) have little relevance to glycolytic flux or growth rate, since xylose fermentation was completed by KO11 within 48 h and the growth ceased for both strains after approximately 24 h.

**Cluster analysis to define corresponding physiological states.** Many differences exist between strains KO11 and B, such as growth rates, glycolytic flux, and fermentation products (Fig. 2 and 3). Cluster analysis was used to identify the most appropriate pairing of samples for comparison assuming that equivalent physiological states are similar (Fig. 4A). Samples clustered into three groups for both strains, in agreement with growth state. (Bootstrap analysis of the validity of groupings at each node is also shown in Fig. 4.) Growing cells during the early stages of fermentation (6 and 12 h) formed one group. The latter stages of fermentation (24 and 48 h) formed a second group. Samples from the end of fermentation (72 h) formed the third group. Expression profiles for KO11 and strain B were also clustered together at 36 h, but this cluster did not form the expected associations between adjacent time points. Based on cluster analyses, pairwise comparisons of the two strains are appropriate for the 6-, 12-, 24-, and 72-h samples. Comparisons at 36 h appear valid but may be anomalous.

**Expression of genes involved in the metabolism of xylose to pyruvate.** Figure 5 summarizes the relative expression levels for 30 genes involved in the catabolism of xylose to pyruvate. Although results at each time point were normalized for total bound cDNA, higher values were observed for most of these genes during active growth and high glycolytic flux (6, 12, and 24 h) than during the latter stages of fermentation (36, 48, and 72 h). For many genes, expression levels declined to 20% of initial values during the later stages of fermentation. For further analysis, genes were divided into three groups: (i) genes unique to xylose catabolism (Fig. 5A), (ii) genes common to pentose metabolism (Fig. 5B), and (iii) genes common to the Embden-Meyerhoff pathway (Fig. 5C). With the exception of *tklA*, the combined cellular allocation of mRNA to the six xylose-specific enzymes exceeded the sum allocated to 22 enzymes in the other two groups (two regulatory genes). The *tklA* gene was expressed at very high levels in both strains, approximately 1% of total cDNA for all samples throughout fermentation. Only six genes involved in xylose metabolism were expressed at a level exceeding 0.1% of total bound cDNA: *xylA*, *xylB*, *xylE*, *tklA*, *gapA*, and *eno*. Despite differences in xylose concentration, fermentation products, glycolytic flux, and growth rate, expression trends exhibited by many of these genes during fermentation were quite similar for KO11 and strain B.

Some genes could be grouped by similarities in expression patterns using visual inspection. As expected, genes in the three *xyl* operons (*xylAB*, *xylE*, and *xylFGH*) followed a similar trend in each strain consistent with regulation by xylose and the xylose regulatory protein (XylR). It is interesting that *gapA* and *pgk* (both monocistronic in *E. coli*) also exhibited similar trends. In some bacteria, *gap* and *pgk* are part of a single operon (6). Cluster analysis was used to provide a more quantitative evaluation of similarities in patterns of gene expression. An initial analysis (data not shown) was performed using a group of 402 genes based on the classification by Riley and Labedan (28). This group included genes encoding carbon metabolism, energy metabolism, and central and intermediary metabolism, *yibO* (phosphoglycerate mutase), *fbaB* (aldolase I, b2097), *xylE* (xylose symporter), and *xylFGH* (xylose ABC transporter). Four clusters of interest were identified. Three xylose genes (*xylE*, *xylF*, and *xylG*) were grouped without additional genes. The remaining four xylose genes (*xylA*, *xylB*, *xylH*, and *xylR*) were clustered with two other genes (bgIB and malS). Both *fbaB* and *talA* were included in a small cluster of four genes with *msgA* and *dsdA*. Eight genes (*tpiA*, *gapA*, *pfkB*, *eno*, *talB*, *pgk*, *pykF*, and *fbaA*) involved in later steps of xylose catabolism were clustered with 17 other genes (primarily *nuo* and *atp* genes). The remaining 13 genes were scattered individually.

Figure 4B shows a cluster analysis of the genes involved in the catabolism of xylose to pyruvate. Euclidean bootstrap analysis was used to evaluate the significance of clustering and confirmed the assignment of most genes to one of three expression patterns during fermentation. Group 1 consisted of five genes (*gapC_1*, *gapC_2*, *gpmA*, *fbaB*, and *talA*). Group 3 consisted of three xylose genes (*xylE*, *xylF*, and *xylG*). Group 2 was the largest and was further divided into two prominent subgroups. One subgroup contained *xylA*, *xylB*, *xylR*, and *pfkB*. The second subgroup contained the eight genes (*tpiA*, *gapA*, *pfkB*, *fbaA*, *pykF*, *pgk*, *talB*, and *eno*) identified in the initial cluster analysis, plus *xylH* and *pykA*. Two genes, *yibO* and *tklB*, were not closely related to any other gene. During fermentation (Fig. 5), relative expression levels for the *tklB* gene re-
Gene expression during xylose fermentation remained essentially constant while those for *yibO* increased with fermentation time.

**Clues to higher levels of glycolytic flux from mRNA profiles.** Most of the genes involved in xylose metabolism were judged to be expressed at higher levels in KO11 than in strain B during active fermentation (6, 12, and 24 h). Increases in expression levels of 14 genes were significant (*P*, 0.05) and are marked by open boxes in Fig. 5. Genes that were higher in KO11 included *xylR* and all *xylR*-regulated genes (*xylA, xylB, xylE, xylF, and xylG*) except *xylH*, two genes in pentose metabolism (*rpiA* and *rpiB*), and six genes in the Embden-Meyerhoff pathway (*pfkB, fbaA, tpiA, gapA, pgk*, and *pykF*). Four genes (*eno, talA, fbaA*, and *fbaB*) were significantly higher in strain B than KO11 at single time points, although differences in expression levels were generally small. None of the genes involved in xylose catabolism was expressed at a significantly higher level in KO11 than strain B after the completion of fermentation (48 or 72 h). Most of the significantly higher values for KO11 genes were from the 6-h samples, the time at which growth rate and glycolytic flux were highest. This is in contrast to the comparison of 4,080 ORFs, where 8% were significantly higher in KO11.

**Comparison of changes in glycolytic flux to changes in gene expression.** The increase in expression levels of catabolic genes in KO11 (compared to levels in strain B) would be expected to result in higher specific activities for corresponding enzymes and may provide a physiological basis for the higher rates of xylose metabolism and growth in KO11 than in strain B. Val-
ues for glycolytic flux in KO11 and strain B were also included in Fig. 5A to facilitate a comparison to gene expression. During fermentation, expression levels in both strains changed coordinately with glycolytic flux for \textit{xylA}, \textit{xylB}, \textit{pfkB}, \textit{talB}, \textit{fbaA}, \textit{gapA}, \textit{pgk}, \textit{eno}, \textit{pykA}, and \textit{pykF}. For many genes, both flux and average expression levels were higher in KO11 than in strain B during the early stages of fermentation and declined in both strains at later times. For some genes, the correlation between flux and expression for data pooled from both strains can be approximated by a single line (Fig. 3C). One or more of these
genes may limit glycolytic flux. None of the genes involved in the catabolism of xylose to pyruvate exhibited an abrupt change in expression similar to that observed for growth rate (Fig. 3A).

**Comparison of mRNA ratios and specific activities for xylA and xylB.** Two inducible enzymes, xylose isomerase and xylulokinase, catalyze initial steps specific to the metabolism of xylose. The expression levels of the corresponding genes (xylA and xylB, respectively) were higher in KO11 than in strain B and roughly correlated with changes in glycolytic flux. However, enzymatic activities rather than mRNA levels determine the rate of metabolic processes. Table 1 shows a comparison of specific activities for xylose isomerase and xylulokinase in both strains at various times during batch fermentation. In general, specific activities for both enzymes were highest at 6 h and declined by less than 50% during fermentation. The specific activities for both enzymes were consistently higher in KO11 than in strain B at all sample times. Specific activities for both enzymes were similar to the maximum glycolytic flux at 6 h and suggest that xylose isomerase and xylulokinase may not be present in excess.

Enzyme activity ratios between KO11 and strain B for xylose isomerase and xylulokinase were compared to corresponding ratios of gene expression, with reasonable agreement. Both ratios were higher in KO11 than in strain B at all time points. During the growth phase (6 and 12 h), enzyme ratios for KO11 and strain B were lower than the expression ratios. During the latter stages of fermentation (36 and 48 h), enzyme ratios were higher than expression ratios. The average ratios for all time points were the same for both specific activities and cDNA-based measurements of gene expression. Activities for xylose isomerase and xylulokinase remained high after growth had ceased, indicating that both enzymes were stable and not rapidly degraded.

The specific activities of xylose isomerase and xylulokinase were used to estimate the cellular content of these two enzymes as a percentage of total cellular protein (100 × specific activity of cell extract/specific activity of pure enzyme). Purified *E. coli* xylose isomerase (recombinant) has been reported to have a maximum activity of 0.87 U mg of protein⁻¹ (30), an unusually low value which may include misfolded protein. For our calculations, a more typical value of 10 U mg of protein⁻¹ (7, 18) was used. This enzyme was estimated to constitute 0.2% glucose were included for comparison (36). With the possible exception of *fbaA* and *fbaB* (higher *fbaA/fbaB* expression ratio in KO11), the relative expression ratios for each set of genes encoding isoenzymes were identical in KO11 and strain B. Although many of these patterns were similar to those in aerobically grown MG1655, the two genes encoding ribose-5 phosphate isomerases (*rpiA* and *rpiB*) were expressed at equal levels in KO11 and strain B, in contrast to MG1655, where *rpiA*...
DISCUSSION

Higher mRNA levels of genes involved in xylose metabolism provide a physiological basis for the increase in growth rate and glycolytic flux in ethanologenic KO11. The metabolic engineering of strain B for ethanol production was accompanied by an increase in growth rate, glycolytic flux (xylose), and the calculated rate of substrate level phosphorylation. The conservation of energy is quite limited during xylose fermentation as illustrated in Fig. 1. Six xylose molecules are transformed into 10 trioses that are metabolized to produce 10 molecules of NADH, 10 molecules of pyruvic acid, and 20 molecules of ATP. Correcting for the estimated energy requirement for xylose uptake (~6 ATP molecules) and sugar activation (~10 ATP molecules), the net energy conserved during xylose metabolism is estimated to be ~0.67 molecule of ATP per xylose molecule, less than half that produced from glucose (~2 ATP molecules/glucose molecule). Thus, ATP production may serve to limit growth rate during xylose fermentation even in rich media. This limitation was partially overcome by the increase in glycolytic flux that accompanied expression of the genes encoding Z. mobilis pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adhB).

The increase in glycolytic flux (and rate of substrate level
phosphorylation) in KO11 appears to result from increased expression of many genes involved in the metabolism of xylose to pyruvate. The most significant increases occurred with genes specific to xylose metabolism. The positive regulator XylR and five of the six enzymes encoded by xyl genes were expressed at significantly higher levels in KO11 than in strain B. The three- and twofold-higher levels of xylA and xylB messages in KO11 were in agreement with measured ratios of specific activities for xylose isomerase and xylulokinase, respectively. Previous studies by Arfin et al. (1) with E. coli and by Zeeman et al. (42) with Kluyveromyces lactis have also reported agreement between changes in mRNA expression levels and changes in enzymatic activities. Expression of two genes in ribose metabolism (pka and talB) and seven genes common to the Embden-Meyerhoff pathway was also significantly higher (P < 0.05) in KO11 during active fermentation (6, 12, or 24 h). Changes in the expression level of 10 genes (xylA, xylB, pfkB, talB, fbaA, gapA, pgk, eno, pykA, and pykF) correlated with changes in glycolytic flux in KO11 and in strain B. For at least four of these genes, pooled data from both strains show a linear relationship between expression level and glycolytic flux (Fig. 3C). Since increased mRNA levels would be expected to result in increased specific activities as shown for xylA and xylB, the 11 activities encoded by these genes may share the control of flux during xylose metabolism. Elevated levels of these enzymes in KO11 are presumed to provide a physiological basis for the observed increase in glycolytic flux and growth rate.

No regulatory protein is known that coordinates the activities of these 11 enzymes. The xylAB and xylFGH operons are under the positive control of xylR (32). Both operons are inducible and were expressed at 50- to 100-fold-higher levels in KO11 and strain B during fermentative growth on xylose than in strain MG1655 during aerobic growth on glucose (36). Increased expression of xylAB in KO11 could result from the observed increase in xylR message. Seven genes common to the Embden-Meyerhoff pathway (pfkB, fbaA, gapA, pgk, eno, pykA, and pykF) also changed in a coordinated manner during fermentation in KO11 and strain B, consistent with some form of regulation. It is tempting to view the changes in expression levels for seven Embden-Meyerhoff enzymes and the xyl operons as adaptive in nature, responding to needs dictated by metabolism. These changes in mRNAs may represent a programmed cellular response to changes in the concentration of cofactors or metabolic intermediates imposed by overexpression of Z. mobilis genes encoding alcohol dehydrogenase and pyruvate decarboxylase. The Z. mobilis pyruvate decarboxylase Kₚ for pyruvate is over 20-fold lower than of the E. coli lactate dehydrogenase, the dominant route for NADH oxidation in the parental strain.

How many ORFs are expressed in E. coli B and KO11 during fermentation? Previous reports of gene expression in E. coli have estimated various numbers of expressed ORFs in E. coli using different methodologies (1, 11, 22, 27, 36). Estimates for cells during rapid aerobic growth have ranged from 25% (27) of the predicted ORFs in the first published paper to 60% (1) of the predicted ORFs in the most recent publication. These differences may be due in part to differences in methodology, and to progressive improvements in software for quantitation and background correction. Although we have no unequivocal method to determine the lower threshold at which an ORF can be regarded as expressed, values below 0.001% of total bound cDNA were not reliably measured and thus form a lower limit. Of the 4,290 predicted ORFs, the averaged values for 95% of these (4,080 ORFs) exceeded 0.001% of bound cDNA. At twice this threshold level, the number of genes with higher values was 58% at 6 h and 77% at 22 h. It is possible that these threshold values are too low, since they would imply expression of almost twice the predicted number of expressed ORFs for E. coli (22). It is certainly expected that the low values observed for some of the ORFs will be found to result from cross-hybridization rather than expression. However, it is also possible that many more proteins are produced in E. coli than originally predicted. Recent studies by ter Linde et al. (37) have reported the expression of 5,738 of the 6,171 ORFs in S. cerevisiae, 93% of the proposed ORFs.

Detection methods aside, the proportion of message for an average gene should approximate the proportion of corresponding protein during steady state, despite differences in translational and posttranslational processes for some gene products. In Z. mobilis, mRNA abundance and mRNA stability were directly correlated with protein levels for many glycolytic enzymes (6, 21). Assuming that E. coli contains 2,350,000 protein molecules per cell (22), the lower threshold for measurements of mRNA abundance as bound cDNA (0.001% of total protein, mass), or 24 protein molecules of average size (Mₚ, 40,000) per cell. Assuming three to four rounds of translation for each message, this lower limit for detection using cDNA (0.001% of total) is estimated at six to eight RNA molecules per cell.

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