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Global Expression Profiling of Acetate-grown *Escherichia coli**

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This study characterized the transcript profile of *Escherichia coli* in acetate cultures using DNA microarray on glass slides. Glucose-grown cultures were used as a reference. At the 95% confidence level, 354 genes were up-regulated in acetate, while 370 genes were down-regulated compared with the glucose-grown culture. Generally, more metabolic genes were up-regulated in acetate than other gene groups, while genes involved in cell replication, transcription, and translation machinery tended to be down-regulated. It appears that *E. coli* commits more resources to metabolism at the expense of growth when cultured in the poor carbon source. The expression profile confirms many known features in acetate metabolism such as the induction of the glyoxylate pathway, tricarboxylic acid cycle, and gluconeogenic genes. It also provided many previously unknown features, including induction of malic enzymes, *ppsA*, and the glycolate pathway and repression of glycolytic and glucose phosphotransferase genes in acetate. The carbon flux delivered from the malic enzymes and PpsA in acetate was further confirmed by deletion mutations. In general, the gene expression profiles qualitatively agree with the metabolic flux changes and may serve as a predictor for gene function and metabolic flux distribution.

Physiological characteristics of *Escherichia coli* using acetate or glucose as a sole carbon and energy source have been studied for more than three decades (1, 2). Briefly, *E. coli* uptakes glucose using the phosphotransferase system that converts extracellular glucose into intracellular glucose 6-phosphate, which can be further metabolized by the glycolytic pathway to produce energy and biosynthetic precursors. In the presence of glucose, the adenylate cyclase is inactive, and the cAMP level is low. In the absence of glucose, the adenylate cyclase is activated to produce cAMP, which when binding to the cAMP receptor protein activates the expression of a large set of catabolite derepression genes (2, 3). On the other hand, acetate is transported into the cell and converted to acetyl-CoA, which is further metabolized through the glyoxylate shunt and the tricarboxylic acid cycle. The acetate-metabolizing genes are typically repressed in the presence of glucose. The induction and regulation of acetate-metabolizing genes have been studied extensively (4). Since the two carbon sources, glucose and acetate, are utilized by distinct metabolic pathways, the metabolic flux distribution differs significantly in these two carbon sources (5). Understanding global gene expression profiles in different carbon sources is important to the investigation of *E. coli* growth in natural environment, where the availability of carbon sources changes dynamically. Acetate-metabolizing culture is particularly relevant to the biotechnology industry, since the accumulation of acetate in bioreactor is commonly observed and often poses as an obstacle to high cell density cultivation (6).

The recent advent of microarray technology allows a thorough analysis of gene expression patterns in different environmental conditions (7, 8). In this approach, individual DNA probes are arrayed on a small glass surface, and labeled first strand cDNA from specific tissue or cell sources is hybridized onto the array. The amount of fluorescence at each DNA probe spot correlates with the abundance of specific mRNA transcript in the cell. This approach enables the characterization of transcriptionally regulated pathways at a genomic scale. In particular, the genome of *E. coli* has been arrayed and used for the comprehensive analysis of the expression level in various physiological states (9–21).

In this paper, we investigated the transcript profile of *E. coli* grown in acetate as the sole carbon source and compared it with the culture grown in glucose. We employed the fluorescence-based microarray system by spotting 96% *E. coli* open reading frames on glass slides. The expression levels of each gene were monitored by fluorescence-labeled cDNA using the printed DNA microarray. A rigorous statistical method was used to access the confidence interval of expression ratio of each gene by taking into account slide-to-slide and culture-to-culture variations. The gene expression profile in acetate was used to assess the metabolic flux distribution in key pathways.

EXPERIMENTAL PROCEDURES

Preparation of DNA Array—To make the *E. coli* cDNA microarrays, PCRs were performed in 96-well plates using Genosys *E. coli* ORFmers (Sigma) as primers and *E. coli* MG1655 (*E. coli* Genetic Stock Center, Yale University) chromosome as a template. Eppendorf MasterTaq Kit (Westbury, NY) was used for a 100-μl volume PCR. After PCR, 3 μl of PCR solutions were run in 1% (w/v) agarose gel to examine the quality of PCR products. Among 4290 primer pairs, 161 pairs failed to yield the desired PCR products. The remaining 4129 PCR products representing 96% of the *E. coli* open reading frames were precipitated by mixing with 10 μl of 3 M sodium acetate (pH 5.3) and 66 μl of isopropyl alcohol and centrifuging at 4000 rpm for 45 min. The precipitants were dissolved in 100 μl of TE buffer (pH 8.0) and then precipitated once again with isopropyl alcohol. After they were dried overnight, the precipitants were dissolved in 10 μl of 350 mM sodium bicarbonate/carbonate buffer (pH 9.0) and printed on a poly-L-lysine-coated glass slide using a robotic spotter. The diameter of each spot was about 150 μm, and the distance between the centers of the spots was about 250 μm.

The slide was hydrated over 95 °C water for 5 s and snap dried on a 100 °C heating block. The probes were cross-linked to the surface of the slide by UV light using the Stratalinker (Stratagene, La Jolla, CA) at 400 mJ. The free lysine groups on the slide surface were blocked by soaking slides in the mixture of 315 ml of *m*-methylpyrrilidinone with 5 g of succinic anhydride and 35 ml of sodium borate solution (0.2 M, pH 8.0) for 15 min. The slides were then washed with 95 °C water for 2 min

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and transferred to 95% ethanol at room temperature for 1 min. The slides were dried by centrifugation (22).

Strain and Culture Conditions—E. coli MC4100 (F⁻*araD139 (argF-lac) U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR*) was cultured in M9 minimal medium (23) containing either 0.5% (w/v) glucose or 0.25% (w/v) acetate as carbon sources for the steady state experiment. 125 mg/liter (w/v) of arginine was added to the medium. When an OD of the cell reached 0.4–0.6 at 550 nm, the cultures were quickly chilled in an ethanol/dry ice bath and harvested by centrifugation for RNA purification.

RNA Purification and Labeling—Total RNA was purified from roughly 1×10^9 cells using the RNeasy Midi Kit (Qiagen, Valencia, CA) by following the manufacturer's instructions. The RNA solution was incubated at 37 °C with 100 units of DNase (Invitrogen) and 40 units of RNAsin ribonuclease inhibitor (Promega, Madison, WI) for 30 min, extracted with phenol/chloroform, and then precipitated with 2.5 volumes of ethanol. After dissolved in 10–20 μl of RNase-free water, 30 μg of total RNA was labeled with either Cy3 or Cy5 dCTPs during reverse transcription. The reverse transcription mixture included 200 units of Superscript RNase H⁻ reverse transcriptase (Invitrogen), random hexamers (Invitrogen), 0.5 mM dATP, dTTP, and dGTP, 0.2 mM dCTP, and 0.1 mM Cy3 or Cy5 dCTP (Amersham Biosciences). After reverse transcription, the RNA was degraded by incubating at 65 °C for 40 min after adding 2 μl of 0.5 M EDTA (pH 8.0) and 5 μl of 1 N NaOH. The cDNAs, labeled with either Cy3 or Cy5, were diluted with 60 μl of TE buffer (pH 8.0) and then mixed together. The labeled cDNA mixture was then concentrated to 1–2 μl by using Micron-50 from Millipore Corp. (Bedford, MA).

Hybridization and Scanning—The concentrated Cy3 and Cy5 cDNA was resuspended in 10 μl of hybridization solution, consisting of 50% formamide, 3× SSC, 1% SDS, 5× Denhardt's solution, 0.1 mg/ml salmon sperm DNA, 0.05 mg/ml yeast total RNA. The labeled cDNA in hybridization solution was denatured in 95 °C for 2 min and cooled for 5 min at room temperature. The hybridization solution was then placed on the slide and covered by cover glass. The slide was assembled with a hybridization chamber (Corning) and hybridized for 14–20 h at 42 °C inside a water bath. The slide was washed in 2× SSC, 0.1% SDS for 5 min at room temperature and then 0.2× SSC for 5 min prior to scanning.

After it was dried by centrifugation, the hybridized slide was scanned with an Affymetrix GMS-418 scanner (Santa Clara, CA). The two images with the wavelengths of Cy3 and Cy5 dyes were individually analyzed by use of image processing software, Imagene (Biodiscovery, Santa Monica, CA). The median intensities of each spot calculated by the program were obtained for further analysis.

Internal Normalization by Rank-invariant Method—It has been shown that the intensity of Cy3 and Cy5 labeling is different and that the correlation between Cy3 and Cy5 intensity is slide-dependent (24). Thus, each slide needed an internal normalization to account for the labeling effect. We used a rank-invariant criterion to select genes that were nondifferentially expressed in each slide (24). These genes were then used to determine the normalization curve. This method was based on the assumption that if a gene is up-regulated, its intensity rank among one channel, say Cy5, should be significantly higher than the rank among the other. This method may fail in some extreme cases where a majority of genes are up-regulated (or down-regulated) to the same extent. However, if there are a large number of nondifferentially expressed genes, as in the case of most cDNA microarray experiments, this method works well.

The ranks of Cy3 and Cy5 intensities of each gene on the slide were separately computed. For a given gene, if the ranks of Cy3 and Cy5 intensities differ by less than a threshold value and the rank of the averaged intensity was not among the extremely high or low, this gene was classified as a rank-invariant gene. We used an iterative selection scheme to achieve this task. In each iteration, the threshold for rank difference was determined by the number of remaining genes multiplied by a predetermined percentage, and the threshold for rank averaged intensity was only applied in the first iteration. The iteration stopped when the remaining set of genes did not decrease in the selection criterion. After the selection of the rank-invariant genes, a quadratic equation was used to fit the data. After normalization, the logarithmic residuals were calculated as $\log(Cy5_i/f(Cy3_i))$, where $Cy3_i$ and $Cy5_i$ represent Cy3 and Cy5 intensities on spot i, and $f(x)$ is the normalization function between the two channels.

Confidence Interval of the Gene Expression Ratios—We previously developed a statistical method for computing the confidence intervals of each gene (24). This method considers the slide-to-slide and culture-to-culture variations. Calibration experiments, where Cy3- and Cy5-labeled cDNAs from the same culture were pooled together and co-hybridized to multiple slides, were used to assess the between-slide variations. The culture-to-culture variation was assessed by repeating the same experiment multiple times under the same condition. With such data, we then used a hierarchical Bayesian model and a Markov chain Monte Carlo (MCMC)[1] simulation to determine the confidence intervals of the gene expression ratios. The details of this method have been described previously (24). A computer program was developed for such a simulation, which is available upon request.

Monitoring ppsA Promoter Activity Using Promoter Fusion to Green Fluorescent Protein—The gene coding green fluorescent protein was amplified from pGFPuv (CLONTECH, Palo Alto, CA) and cloned in *Eco*RI and *Hin*dIII restriction sites of pJF118EH (25) to form pMK50. The *ppsA* promoter region was amplified by the primers 5′-CGGAGCTCGCACAGAAGCGTAGAACG-3′ and 5′-CGAATTCCTTTTGTGATAAATGAACGG-3′ from the *E. coli* chromosome and cloned into the *Eco*RI and *Eco*RV sites of the pMK50. The resulting plasmid is named pKK1. The plasmid was transformed into MC4100. Fluorescence intensity was measured using a fluorimeter (ISA, Inc., Edison, NJ) during exponential growth phase (A_{600} = 0.3–0.8) in M9 minimal medium with glucose or acetate. Fluorescence was excited at a wavelength of 395 nm, and emission was measured at a wavelength of 509 nm. The fluorescence intensity for each culture was normalized by dividing the fluorescence by cell density at A_{600}.

Deletion Mutation of pckA, ppsA, sfcA, and maeB—Each gene was disrupted by the method developed by Datsenko and Wanner (26). Briefly, primers (listed in Table I) for deletion were used to amplify the chloramphenicol-resistant gene from pKD3 (26). Strains harboring pKD46 (26) were grown in SOB medium containing 200 mg/liter ampicillin and 1 mM L-arabinose and transformed with the PCR products using an electroporator (Eppendorf). Chloramphenicol-resistant strains were selected on agar plate, and the chloramphenicol-resistant gene was eliminated from the strain by transforming pCP20 (26) and colony-purified at 42 °C. The chloramphenicol-resistant gene and pCP20 were popped out during this process. The mutation was confirmed by PCR with primers for confirmation (Table I). Multiple deletion mutations were performed sequentially.

RESULTS

Transcript Profiling Using DNA Microarray—E. coli transcription profiles in acetate and glucose minimal media were compared using six sets of DNA microarray data generated from three independent experiments. To achieve a balanced physiological state, the *E. coli* strain was subcultured at least twice after initial inoculation from an LB agar plate and harvested in the midlog phase. Total RNA was purified and labeled with Cy3 or Cy5 dCTP during reverse transcription. In the first two experiments, we labeled the RNA from the glucose culture with Cy5 dyes and the RNA from the acetate culture with Cy3. The labels were reversed in the third experiment. In each experiment, the labeled cDNAs were mixed and then hybridized on two microarray slides. For calibration experiments, RNA from glucose culture was divided into two tubes and labeled with Cy3 and Cy5 dCTP, respectively, and hybridized to two slides. The same process was repeated with RNA purified from an acetate culture. Four data sets of calibration experiments were used to provide the statistical parameter for slide-dependent variation of each gene. This information was incorporated to evaluate the confidence intervals in the MCMC method.

The arraying solutions without DNA were arrayed 24 times on each slide in four different positions and used as negative controls. The average plus two S.D. values of intensities of the negative controls was computed in every slide and used as a threshold for data filtering. The spots showing intensity lower than the threshold were filtered out. These spots were attributed to either improper probe arraying or low expression. The spots whose intensities exceeded the detection range of the scanner were also excluded. After filtering the outliers, the rank-invariant genes were selected as a basis for normalization, and

[1] The abbreviation used is: MCMC, Markov chain Monte Carlo.

TABLE I
The primer sequences used to delete the gluconeogenic genes

Gene	Primers for deletion (5′ to 3′)
ppsA	gcgcaacgctgggatcagtcttaaaaagtaaaaaaatatatttgcttgaagtgtaggctggagctgcttc
	ttcatcttcggggatcacataacccggcgactaaacgccgccggggatttcatatgaatatcctccttag
pckA	gagccttgtcgcggttaacacccccaaaaagactttactattcaggcaatgtgtaggctggagctgcttc
	gtcgataaacagtttcgccagggtttcggctttttcctgccactgttccgcatatgaatatcctccttag
sfcA	ctttatacttgaggccgacgccctggcggtaaagcaaagacgataaaagcgtgtaggctggagctgcttc
	cgcaggcttagatggaggtacggcggtagtcgcggtattcggcttgccagcatatgaatatcctccttag
maeB	ttgtgaacgttacgtgaaaggaacaaccaaatggatgaccagttaaaaacagtgtaggctggagctgcttc
	cggtttcgccacacccatcagcaccgggccgacagtcacaccttccgagccatatgaatatcctccttag
	Primers for confirmation (5′ to 3′)
ppsA	aacagcattatccatttgttcttc
	gatctctgccgtatggatgag
pckA	aattatcgcatccgggcag
	cagggcacgacaaaagaag
sfcA	acaccacaacactccattttc
	gctatgcttgatcggcaac
maeB	ggatgataatggcgaatggac
	aattacagcggttgggtttg

then the logarithmic ratio of expression levels were calculated as described above. The four sets of calibration data and six sets of acetate/glucose comparison data were used to access the expression ratios using the MCMC method. The expression levels of 3649 genes, which passed the threshold filtering, were computed successfully. Among them, 354 genes were up-regulated, and 370 genes were down-regulated with 95% confidence in acetate medium compared with those in glucose medium. Because of the high number of simultaneous statistical tests used, the error rate at 5% for 4000 genes will generate 200 false positives when discussing the genome as a whole. Therefore, a more stringent confidence level was also used. With 99% confidence, 185 and 177 genes were up- and down-regulated, respectively. At this confidence level, the error rate of 1% will produce 40 false positives. However, when discussing individual genes, a 95% confidence level was appropriate for most purposes discussed here unless specified otherwise.

In Fig. 1, the logarithmic ratio of each gene was plotted against the average log intensity of that gene. The *red* and *green dots* represent the genes that were up- or down-regulated, respectively, with 95 or 99% confidence, whereas the *black dots* represent the genes that were not differentially regulated based on the statistical judgment. Fig. 1 shows that the -fold difference does not necessarily correlate with statistical significance, as previously pointed out (10). For example, *rpmF* was down-regulated more than 4-fold in acetate, but it was statistically insignificant because of its high variances of the data. Meanwhile, the *dnaN* level was repressed only 1.3-fold with very small variance, and thus this gene was determined to be down-regulated with a 99% confidence level. The source of the variance can be severalfold, including poor quality or quantity of PCR products, cross-contamination by nonspecific binding, and high background on the slides. Therefore, a vigorous statistical analysis and repeats of the experiment are essential to obtain reliable data. The expression ratios and confidence intervals of all the genes are available on the World Wide Web at www.seas.ucla.edu/~liaoj/glcace.html.

Functional Groups of Differentially Regulated Genes—The differentially expressed genes with 95 and 99% confidence intervals were further classified into 23 groups by their functions (Table II). As expected, the most significant difference of growing in different carbon sources occurred among the central intermediary metabolic genes. 27 and 14% of the 161 genes in this group were significantly induced or repressed, respectively, in acetate medium compared with those in glucose medium. In the functional groups, such as carbon compound

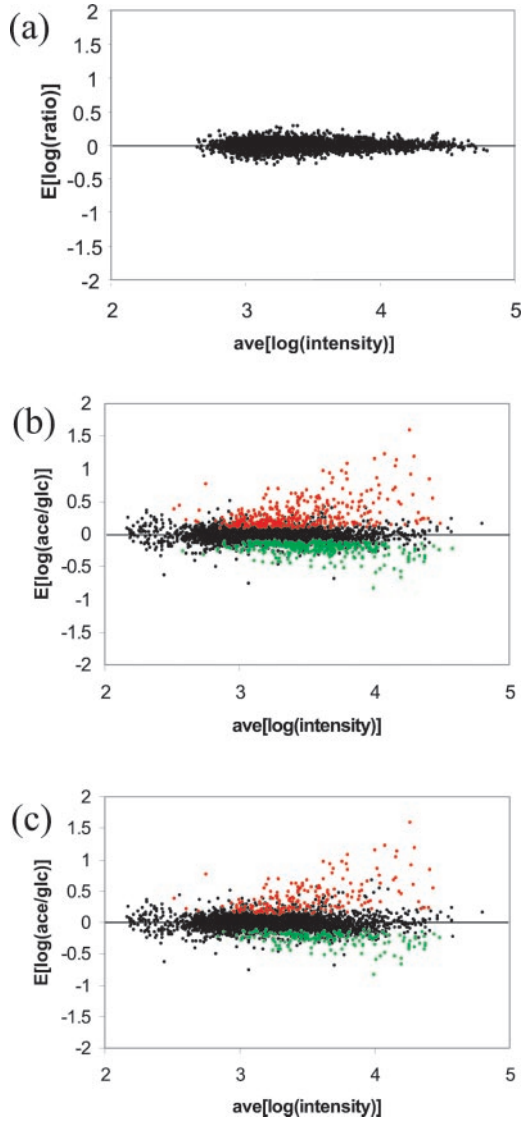

FIG. 1. **Expected logarithmic ratios between two dyes (*y* axis) in calibration experiments (*a*) and glucose-acetate comparison experiments (*b* and *c*) were plotted against log-transformed mean fluorescence intensities of the genes.** The *red dots* represent up-regulated genes, and *green dots* show down-regulated genes with 95% confidence level (*b*) and 99% confidence level (*c*).

TABLE II
Numbers and percentages of up- or down-regulated genes in each functional group
Annotations follow Blattner *et al* (50).

Functional groups	95% confidence		99% confidence		Total no. of genes
	Up	Down	Up	Down	
Amino acid biosynthesis and metabolism	9 (7.8%)	28 (24.5%)	4 (3.5%)	21 (18.3%)	115
Biosynthesis of cofactors, prosthetic groups, and carriers	9 (10.0%)	8 (8.9%)	3 (3.3%)	1 (1.1%)	90
Carbon compound catabolism	22 (18.8%)	4 (3.4%)	9 (7.7%)	1 (0.9%)	117
Cell processes (including adaptation, protection)	6 (3.7%)	13 (8.0%)	0 (0%)	6 (3.7%)	162
Cell structure	7 (4.0%)	21 (11.9%)	4 (2.3%)	7 (4.0%)	176
Central intermediary metabolism	43 (26.7%)	23 (14.3%)	27 (16.8%)	12 (7.5%)	161
DNA replication, recombination, modification, and repair	4 (3.7%)	22 (20.2%)	1 (0.9%)	9 (8.3%)	109
Energy metabolism	30 (14.1%)	24 (11.3%)	15 (7.0%)	16 (7.5%)	213
Fatty acid and phospholipid metabolism	10 (20.8%)	3 (6.3%)	9 (18.8%)	1 (2.1%)	48
Hypothetical, unclassified, unknown	127 (10.0%)	85 (6.7%)	67 (5.3%)	32 (2.5%)	1270
Membrane proteins	0 (0%)	1 (8.3%)	0 (0%)	0 (0%)	12
Nucleotide biosynthesis and metabolism	3 (6.3%)	10 (20.8%)	2 (4.2%)	5 (10.4%)	48
Other known genes	1 (4.2%)	1 (4.2%)	1 (4.2%)	0 (0%)	24
Phage, transposon, or plasmid	14 (17.7%)	2 (2.5%)	5 (6.3%)	1 (1.3%)	79
Putative chaperones	0 (0%)	0 (0%)	0 (0%)	0 (0%)	6
Putative enzymes	28 (12.6%)	15 (6.8%)	16 (7.2%)	8 (3.6%)	222
Putative regulatory proteins	4 (3.3%)	4 (3.3%)	3 (2.5%)	1 (0.8%)	120
Putative transport proteins	4 (3.0%)	12 (9.1%)	2 (1.5%)	4 (3.0%)	132
Regulatory function	1 (2.3%)	6 (14.0%)	0 (0%)	1 (2.3%)	43
Structural proteins	1 (2.5%)	4 (10.0%)	0 (0%)	3 (7.5%)	40
Transcription, RNA processing, and degradation	3 (5.8%)	9 (17.3%)	1 (1.9%)	4 (7.7%)	52
Translation, post-translational modification	2 (1.3%)	47 (31.1%)	1 (0.7%)	28 (18.5%)	151
Transport and binding proteins	26 (10.0%)	28 (10.8%)	15 (5.8%)	16 (6.2%)	259
Total	354 (9.7%)	370 (10.1%)	185 (5.1%)	177 (4.9%)	3649

catabolism and fatty acid metabolism, the numbers of up-regulated genes surpassed those of down-regulated genes significantly, suggesting that these groups of genes were generally repressed by glucose or induced by acetate. Apparently, *E. coli* up-regulates the genes that allow the utilization of any possible carbon sources such as fatty acids when growing in a relatively poor carbon source such as acetate.

On the other hand, the number of down-regulated genes was much higher than up-regulated genes in many functional groups. For example, amino acid biosynthesis and nucleotide biosynthesis genes were generally down-regulated. The number of repressed genes exceeds the induced genes by 28 to 9 and 10 to 3 in these two groups, respectively, suggesting that *E. coli* turned down the expression of biosynthetic genes to match the low growth rate and save energy in the poor carbon source. In addition, in the functional groups such as cell structure, DNA replication, transcription, and translation, the number of down-regulated genes surpasses the up-regulated genes by 21 to 7, 22 to 4, 9 to 3, and 47 to 2, respectively. This result can be explained by the fact that the growth rate was much lower in acetate compared with glucose as a carbon source. Indeed, many of the genes belonging to these categories were known to be correlated with the growth rate of the cell (27).

Central Metabolic Genes Involved in Acetate Metabolism—When acetate is metabolized as a sole carbon and energy source in *E. coli*, it is first activated to acetyl-CoA and then metabolized in the tricarboxylic acid cycle and glyoxylate shunt (4). Two pathways were responsible for the acetate activation, *pta-ackA* and *acs*. Interestingly, *pta* and *ackA* were both down-regulated by ~2-fold in acetate. On the other hand, *acs* was induced more than 8-fold in acetate, one of the most significantly up-regulated genes. This result suggests that Acs is the major enzyme for acetate uptake and activation. Acetyl-CoA is converted to malate through the glyoxylate shunt. The induction mechanism of the glyoxylate shunt genes, *aceB* and *aceA*, in acetate has been well characterized (4). These two genes are located in the same operon with *aceK* (encoding isocitrate dehydrogenase kinase/phosphatase). The expression of *aceB* gene was not monitored in this experiment because of failed PCR amplification. However, the other genes (*aceA* and *aceK*) in the same operon showed more than 10-fold up-regulation. Not only the *aceBAK* operon but also the *glcDFGB* operon was induced significantly. The last gene in the *glc* operon is the secondary malate synthase (*glcB*), which can replace the malate synthase A (*aceB*) in acetate (28) when *aceB* is mutated. This operon could be induced by acetate (29). Meanwhile, most of the tricarboxylic acid cycle genes (*gltA*, *acnA*, *acnB*, *icdA*, *sucABCD* operon, *sdhCDAB* operon, *fumA*, *fumC*, *fumB*, and *mdh*) were up-regulated. In particular, the genes involved in the glyoxylate pathway, *mdh*, *gltA*, and *acnB*, were highly up-regulated more than 4-fold. Together with induction of *aceBAK* operon, these results confirmed that metabolic flux in the glyoxylate cycle (4) is very high in acetate (Fig. 2 and Table III).

The gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (coded by *pckA*), is known to be responsible for delivering the carbon flux from the tricarboxylic acid cycle to the gluconeogenic pathways in acetate (4, 5). Indeed, *pckA* was 5–14-fold induced in acetate. Surprisingly, *ppsA* (coded for phosphoenolpyruvate synthase) was also induced 9–21-fold in acetate, although the gene product was nonessential for gluconeogenesis during growth on acetate (5). This result suggested that PpsA may play an important role for gluconeogenic flux when *E. coli* is grown in acetate. Together with the malic enzymes, PpsA could serve the same function as PckA. Indeed, both NAD-dependent (*sfcA*) and NADP-dependent (*maeB*) malic enzymes were also induced. The functions of these gluconeogenic genes in acetate were further verified with deletion mutations, which will be discussed shortly.

Other Carbon and Energy Metabolism Genes—Many glycolytic genes (*pfkA*, *fba*, *gapA*, *epd*, *pgk*, *eno*, *pykF*, and *ppc*) were down-regulated in acetate. In addition, the first two pentose pathway genes (*zwf* and *gnd*) and pyruvate dehydrogenase (*aceEF* operon) were also significantly down-regulated. These data correlate with the reduced metabolic flux in these pathway genes. The expression levels of many carbon transport genes were also affected seriously by different carbon sources. The genes involved in glucose transport, the *ptsHI-crr* operon and *ptsG*, were repressed significantly in acetate, 1.5–2-fold and 2–4-fold, respectively. These operons were known to be

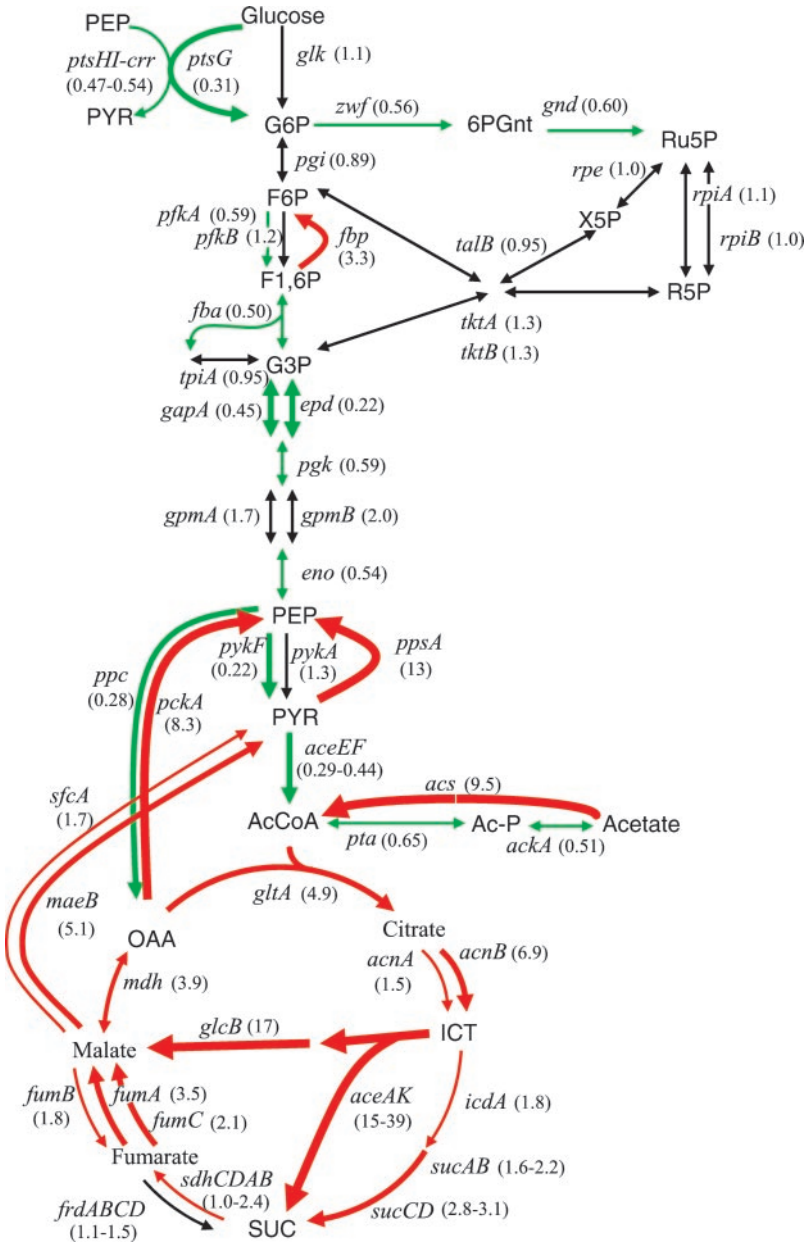

FIG. 2. **The expression levels of central metabolic pathway genes in acetate compared with those in glucose.** The *numbers* beside gene names represent the expected -fold changes of expression levels calculated from six repetitions of experiments. The *red arrows* represent the induced genes, and *green arrows* represent the repressed genes in acetate compared with in glucose with more than 95% confidence. The *thicker* the *arrows*, the higher the genes were regulated. *G6P*, glucose 6-phosphate; *F6P*, fructose 6-phosphate; *F1,6P*, fructose 1,6-phosphate; *G3P*, glyceraldehyde 3-phosphate; *PYR*, pyruvate; *AcCoA*, acetyl-CoA; *Ac-P*, acetylphosphate; *ICT*, isocitrate; *SUC*, succinate; *OAA*, oxaloacetate; *6PGnt*, 6-phosphogluconate; *Ru5P*, ribulose 5-phosphate; *R5P*, ribose 5-phosphate; *X5P*, xylulose 5-phosphate. The gene names followed the *E. coli* K-12 linkage map in Ref. 50.

regulated by *Mlc* (30). In the absence of glucose, *Mlc* represses the operons. In the presence of extracellular glucose, the conformation of $EIIBC^{glc}$ protein is changed and bound strongly with *Mlc*, which no longer represses the operons.

On the other hand, transport genes for other carbon sources were induced significantly by catabolite derepression. Examples are the galactose ABC transporter operon (*mglBAC*, 4–8-fold), the ribose uptake gene operon (*rbsD* and *rbsACB* operon, 2–8-fold and 3–5-fold, respectively), the *N*-acetyl-D-glucosamide transport subunit (*nagE*, 2–4-fold), the arginine ABC transport gene (*argT*, 2–6-fold), the C4 dicarboxylate transporter gene (*dctA*, 3–6-fold) (31), tagatose metaoblic genes (*gatYZ* operon, 3–10-fold), and the maltose translocating gene (*lamB*, 2–4-fold).

Interestingly, not only the genes involved in glyoxylate shunt pathway (*ace* and *glc* operons), but also those involved in other glyoxylate-related metabolic pathways, such as glycolate and allatoine metabolism, were all up-regulated (Fig. 3 and Table IV). These genes are located close together on the chromosome and expressed by four different operons (32). However, the role of these genes in acetate metabolism is unknown.

Genes Involved in the Cell Machinery—Among the genes involved in the cell structure, DNA replication, transcription, and translation, a total of 16 genes (3.3% of the total) were up-regulated, whereas 99 genes (20%) were down-regulated. Generally, the expression data of these groups of genes do not vary much from experiment to experiment compared with those of metabolic genes. Therefore, the accuracy of measurements was higher. Among 60 ribosomal proteins, including S1–S21, L1–L25, L27–L36, two EF-Tu subunits, EF-Ts, and EF-G, the expression levels of 40 genes were successfully monitored. Among them, more than 70% (29 of 40) were down-regulated in acetate at the 95% confidence level. The down-regulation of these genes was attributed to the growth rate-dependent regulation (27).

Roles of Gluconeogenic Genes in Acetate Growth—Although *ppsA* was known to be nonessential for growth in acetate, it was up-regulated 9–21-fold in acetate (Fig. 2 and Table III). To verify the induction of *ppsA* transcript in acetate, we constructed a transcriptional fusion between the *ppsA* promoter and the green fluorescence protein. The *E. coli* MC4100 harboring the reporter plasmid (pKK1) was cultured in either glucose or acetate minimal media, and the fluorescence level

TABLE III

The -fold changes of central metabolic gene expression in acetate compared with glucose

The expected transcript level changes in acetate were between upper and lower bounds when the microarray data were analyzed with 95 and 99% confidence intervals using the MCMC method described under "Experimental Procedures." The -fold changes of operon are the averages of the -fold changes of the genes belonging to the operon.

Gene or operon names	95% confidence		99% confidence		Gene functions
	Lower bound	Upper bound	Lower bound	Upper bound	
ptsHI-crr	0.37	0.7	0.33	0.78	PTS family Hpr, enzyme I and IIA
ptsG	0.21	0.44	0.17	0.52	PTS family enzyme IIC (glucose)
glk	0.74	1.6	0.61	2.1	Glucokinase
pgi	0.57	1.4	0.41	1.7	Glucosephosphate isomerase
pfkA	0.45	0.78	0.38	0.96	6-Phosphofructokinase I
pfkB	0.81	1.9	0.65	2.2	6-Phosphofructokinase II
fbp	2.5	4.3	2.1	5.3	Fructose-bisphosphatase
fba	0.4	0.62	0.33	0.76	Fructose-bisphosphate aldolase
tpiA	0.65	1.4	0.54	1.4	Triose-phosphate isomerase
gapA	0.3	0.69	0.24	1.0	Glyceraldehyde-3-phosphate dehydrogenase A
epd	0.15	0.31	0.14	0.43	D-Erythrose-4-phosphate dehydrogenase
pgk	0.46	0.77	0.41	0.85	Phosphoglycerate kinase
gpmA	0.84	4	0.35	6.6	Phosphoglyceromutase 1
gpmB	0.78	5.1	0.75	6.8	Phosphoglyceromutase 2
eno	0.34	0.86	0.18	0.99	Enolase
pykA	0.98	1.6	0.86	1.8	Pyruvate kinase II
pykF	0.19	0.52	0.14	0.74	Pyruvate kinase I
ppsA	8.6	21.0	6.6	24.7	Phosphoenolpyruvate synthase
ppc	0.17	0.45	0.22	0.53	Phosphoenolpyruvate carboxylase
pckA	4.9	14.4	4.3	15.9	Phosphoenolpyruvate carboxykinase
aceEF	0.27	0.64	0.24	0.77	Pyruvate dehydrogenase
pta-ackA	0.41	0.82	0.34	0.96	Phosphotransacetylase and acetate kinase A
acs	7.8	11.6	6.8	12.6	Acetyl-CoA synthetase
gltA	3.5	6.8	2.9	9.0	Citrate synthase
acnA	1.1	2.1	0.95	2.4	Aconitate hydratase 1
acnB	3.6	13.1	2.0	13.8	Aconitate hydratase 2
icdA	1.3	2.5	1.2	3.2	Isocitrate dehydrogenase
sucAB	1.2	3.2	0.89	4.1	2-Oxoglutarate dehydrogenase
sucCD	1.8	4.7	1.4	5.3	Succinyl-CoA synthetase
sdhCDAB	1.2	2.5	0.88	3.4	Succinate dehydrogenase
frdABCD	0.77	2.2	0.57	2.5	Fumarate reductase
fumA	2.2	5.7	2.0	8.1	Fumarase A
fumB	1.2	2.7	0.9	3.1	Fumarase B
fumC	1.3	3.5	0.78	3.6	Fumarase C
mdh	2.4	6.2	2.0	9.3	Malate dehydrogenase, NAD(P)-binding
aceAK	17.4	32.8	16.1	36.2	Isocitrate lyase and *icdA* kinase/phosphatase
sfcA	1.2	2.4	0.92	2.7	NAD-linked malate dehydrogenase
maeB	4.2	6.4	4.1	7.4	Putative malate dehydrogenase
zwf	0.41	0.77	0.3	0.99	Glucose-6-phosphate dehydrogenase
gnd	0.43	0.84	0.41	0.9	Gluconate-6-phosphate dehydrogenase
rpe	0.78	1.4	0.68	1.5	D-Ribulose-5-phosphate 3-epimerase
rpiA	0.71	1.8	0.54	2.2	Ribosephosphate isomerase
rpiB	0.8	1.4	0.71	1.8	Ribose 5-phosphate isomerase B
talA	1.1	5.2	1.0	7.0	Transaldolase A
talB	0.69	1.3	0.57	1.5	Transaldolase B
tktA	0.95	1.9	0.78	2.5	Transketolase 1
tktB	0.84	2.1	0.67	2.9	Transketolase 2

was monitored in the midlog phase. In two independent experiments, the fluorescence level of the cells grown in acetate was detected at least 4-fold higher than the level in glucose (Fig. 4). This result supports the data obtained by DNA microarray analysis.

Although previously undetermined, the role of PpsA in acetate-grown *E. coli* may be the delivery of metabolites from the tricarboxylic acid cycle to the Embden-Meyerhoff pathway. This gluconeogenic function requires the malic enzymes (coded by *sfcA* and *maeB*), which were also up-regulated in acetate. The malic enzyme-PpsA pathway can theoretically serve as an alternative to PckA, which is known to be the main gluconeogenic pathway in acetate-grown *E. coli* and is also up-regulated significantly. To determine the roles of these genes in *E. coli* growth in acetate, a set of deletion mutants were constructed as described. The deletions of the genes on the chromosome were confirmed by PCR using the primers outside of the genes. The growth rates of those mutants were measured in acetate (Table V). Almost no growth inhibition was detected in either *pckA* or *ppsA* single mutants, while the growth of the *pckA ppsA* double mutant was abolished in acetate (Table V). These results showed that PckA and PpsA-malic enzyme can serve as an alternative pathway to each other and that these are the only two gluconeogenic enzymes to provide the phosphoenolpyruvate pool. We further examined the functions of the malic enzymes, which are required to supply pyruvate, the substrate of PpsA. The deletion mutation of one malic enzyme, either *sfcA* or *maeB*, in the *pckA* background did not reduce the growth rate in acetate. However, the deletion of both malic enzymes with *pckA* mutation abolished the growth in acetate (Table V). The series of mutation studies proved that PckA and PpsA-malic enzymes are the two substitutable pathways that can deliver carbon flux from the tricarboxylic acid cycle to the Embden-Meyerhoff pathway.

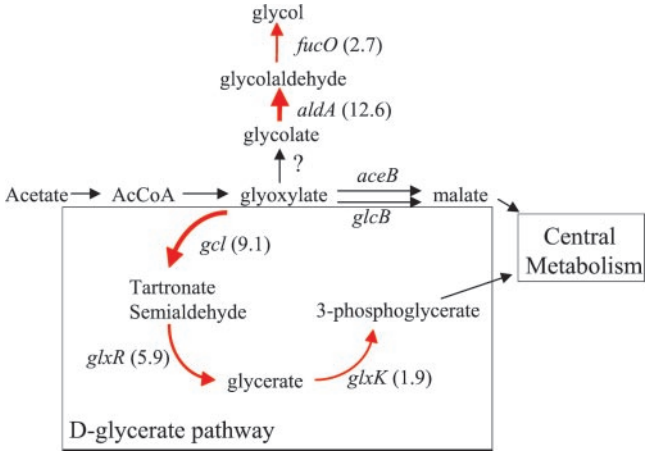

FIG. 3. **The expression profiles of glyoxylate metabolic genes in acetate compared with in glucose.** The *numbers*, *arrows*, and *abbreviations* are as described in the legend to Fig. 2.

DISCUSSION

Statistics—Although DNA microarray has gained momentum in genomic scale expression profiling, methods for assessing statistical significance have just begun to be developed. The initial approach of assigning genes that shows a more than 2-fold expression ratio as "significant" has proven unsatisfactory (10). Because the variations among the signals on the array are gene-dependent, a common threshold for all of the genes is inadequate. Our data here again show the same conclusion. Long *et al.* (33) proposed a *t* test using the Bayesian inference of variance. This approach was developed for the one-channel membrane arrays and used the intensity data of the two treatments as variables for comparison. This method is not optimal for the two-channel glass arrays, which allow the direct comparison of two treatments (acetate and glucose) simultaneously on the same slide. Such two-channel systems reduce the slide-to-slide variation, which can be significant in both glass and membrane arrays. The method used here (24) directly compares the two treatments by taking the log ratio of the normalized intensity. The gene-dependent, slide-to-slide and experiment-to-experiment variations were accounted for in a hierarchical Bayesian model. The gene-specific confidence intervals as a result of multiple experiments and multiple slides were calculated. Because of the large number of statistical tests, we calculated both 95 and 99% confidence levels. At these two confidence levels, about 200 and 40 false positives, respectively, will occur among 4000 genes, when the whole genome is considered simultaneously.

Metabolic Genes—In general, the expression profiles obtained from *E. coli* grown in glucose and acetate agree with the direction of intracellular carbon fluxes. In acetate cultures, the phosphotransferase systems for glucose uptake and glycolytic genes were highly down-regulated compared with those in glucose cultures. On the other hand, genes involved in acetate uptake (*acs*), the glyoxylate cycle (*aceBAK* and *glcB*), the tricarboxylic acid cycle (*gltA*, *icdA*, *acnA*, *acnB*, *sucABCD*, *sdhCDAB*, *fumA*, *fumB*, *fumC*, and *mdh*), and gluconeogenesis (*pckA*, *ppsA*, *sfcA*, and *maeB*) were all up-regulated in acetate. The only exception is the Pta-AckA pathway, which was down-regulated significantly in acetate. This result suggests that Acs is the main path for acetate uptake, whereas the Pta-AckA pathway is used for acetate excretion during growth on glucose. Because the mutation of both *pta* and *ackA* inhibited cell growth in acetate (34), it has been suspected that this pathway also delivers a significant amount of carbon flux into the cell. However, *acs* induction in acetate has been shown to be impaired in the *pta ackA* double mutant (35), which may explain why the *pta ackA* mutant did not grow well in acetate.

Among the genes that are highly up-regulated in acetate, *ppsA* was unexpected, since this gene was dispensable during growth on acetate. The *ppsA* induction was confirmed independently by a promoter fusion experiment. The role of *ppsA* during growth on acetate was demonstrated using mutation analysis. In acetate, PpsA and the two malic enzymes form an alternative pathway to PckA. Deletion mutation of either one of the pathways has no effect on growth rate in acetate, suggesting that either one has sufficient capacity to deliver carbon flux to the Embden-Meyerhoff pathway. The malic enzyme pathway was not clearly defined in *E. coli* previously. It has been known for more than 30 years that two malic enzymes existed in *E. coli* (36). However, the genes coding those enzymes were not mapped on the genetic level until recently. NAD-dependent malic enzyme, *sfcA*, was cloned and characterized recently (37), whereas NADP-dependent malic enzyme, *maeB*, was only predicted with sequence similarity. Interestingly, both *sfcA* and *maeB* were highly up-regulated in acetate. In addition, mutation analysis showed that those genes can compensate each other in acetate cultures.

The most striking difference between acetate-grown and glucose-grown *E. coli* cultures is the up-regulation of metabolic genes such as *acs*, *pckA*, *ppsA*, and *aceBAK*. These genes are either essential or carry significant metabolic flux. In general, results from the microarray analysis summarized in Fig. 2 show a dramatic agreement with the deduced metabolic pathway for acetate growth. All of the essential or flux-carrying pathways (glyoxylate shunt, tricarboxylic acid cycle) were up-regulated, and most of the unused pathways (glycolysis) were down-regulated. It is tempting to suggest that the significantly up-regulated genes delineate the functional pathways. The PpsA-malic enzymes pathway, the PckA pathway, and the Acs pathway are good supports for this argument. If this theory holds, one can predict functional pathways through gene expression profiling.

Regulatory Systems—Although many global regulators have been characterized, it is not straightforward to identify the regulators involved in the change of expression profiles form glucose to acetate cultures. For example, the tricarboxylic acid cycle genes were up-regulated in acetate partly by ArcA and Fnr (38). However, the effects of those regulators were not observed in other ArcA or Fnr-regulated genes, such as *cydAB*, *cyoABCDE*, *dmsABC*, and *sodA* (38). It is possible that multiple regulators co-control these genes to manifest a complex response to the environment. The only regulatory system showing ubiquitous effects on many different genes is the catabolite derepression by cAMP-cAMP receptor protein. This regulator activates many genes in acetate. For example, the tricarboxylic acid cycle and gluconeogenic pathway and many carbon transport genes were fully or partially induced by catabolite derepression. Among the biosynthetic genes, which were generally down-regulated, a few genes, such as *tnaA*, *ilvBN*, *dadX*, and *aspA*, were strongly up-regulated, possibly attributable to this mechanism (39–41). Catabolite derepression also induces fatty acid degradation genes, *fadD* and *fadBA* (42), and flagella regulatory genes (*flhCD*) (43) in acetate growth. Finally, the 11–20-fold and 3–5-fold inductions of *cstA* and *astA*, of which the functions are not clear, were also attributed to this mechanism (44, 45).

Functional Genomics—One of the outstanding features of the microarray experiment is its utility in the prediction of gene functions. In this experiment, the function of *maeB*, the predicted but unconfirmed NADP-dependent malic enzyme, was successfully examined with both microarray and mutation experiments. This gene was up-regulated about 4–6-fold in

TABLE IV

The -fold changes of glycolate metabolic gene expression in acetate compared with glucose

The expected transcript level changes in acetate were between upper and lower bounds when the microarray data were analyzed with 95 and 99% confidence intervals using the MCMC method described under "Experimental Procedures."

Gene name	95% confidence		99% confidence		Gene functions
	Lower bound	Upper bound	Lower bound	Upper bound	
gcl	3.0	28.3	1.0	49.1	Glyoxylate carboligase
glxR	2.3	14.9	2.1	16.9	Tartronic semialdehyde reductase
glxK	1.2	3.0	0.9	4.2	Glycerate kinase II
fucO	1.6	4.4	1.3	4.8	L-1,2-Propanediol oxidoreductase
aldA	6.2	20.4	4.9	29.2	Aldehyde dehydrogenase A, NAD-linked
allA	1.9	8.7	1.2	12.7	Ureidoglycolate hydrolase
allB	3.5	40.3	2.0	93.7	Allantoinase
allC	1.6	2.9	1.5	3.6	Allantoate amidohydrolase
allD	1.3	4.1	1.0	5.5	Ureidoglycolate dehydrogenase
allR	1.6	2.5	1.4	3.0	Allantoin metabolism repressor

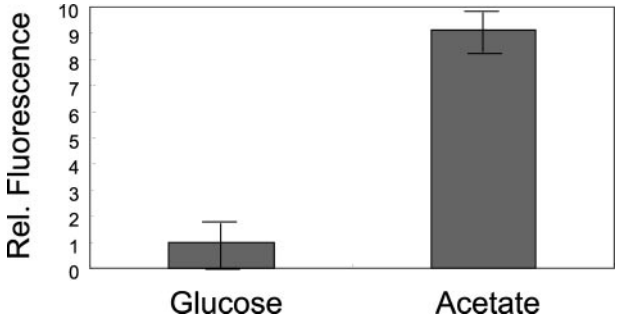

FIG. 4. **The expression levels of green fluorescence protein fused to the *ppsA* promoter in *E. coli* grown in glucose and acetate minimal medium.** The *y* axis is the relative fluorescence level normalized to cell density and the average fluorescence level in glucose.

TABLE V

Growth rates of the mutants of gluconeogenic pathway genes in acetate

The strains were grown in acetate minimal medium, and their growth rates were calculated from the cell density measured every hour.

Strains	Growth rate (h^{-1})
MC4100	0.33 ± 0.05
MC4100 Δ*ppsA*	0.26 ± 0.02
MC4100 Δ*pckA*	0.32 ± 0.02
MC4100 Δ*ppsA* Δ*pckA*	0
MC4100 Δ*pckA* Δ*sfcA*	0.29 ± 0.03
MC4100 Δ*pckA* Δ*maeB*	0.29 ± 0.02
MC4100 Δ*pckA* Δ*sfcA* Δ*maeB*	0

acetate compared with glucose cultures. This result suggested that the *maeB* gene product is involved in acetate metabolism, which is in agreement with the prediction of the gene function (malic enzyme) and successfully confirmed by mutation studies. Another example is the *b2341-b2342* operon, whose function is presumed to be similar to that of the *fadAB* fatty acid degradation genes by the KEGG data base (46). Indeed, both operons were up-regulated 2.5–4- and 2.8–7-fold, respectively, in acetate and have a FadR binding site on their promoters (47).

Meanwhile, the expression level of the newly characterized class I type of fructose-bisphosphate aldolase, coded by *dhnA* (48), showed 3-fold induction in acetate, in contrast to the known fructose-bisphosphate aldolase, *fba*, which was down-regulated 1.5–2.5-fold as other glycolytic genes. This result suggests that the *dhnA* product may serve other functions *in vivo*. Further, the membrane-associated malate dehydrogenase gene, *mqo* (*yojH*) (49), was not regulated in acetate, in contrast to a more than 2–6-fold increase of the other malate dehydrogenase, *mdh*, and up-regulation of other tricarboxylic acid cycle genes. Again, the expression pattern suggests that the *mqo* gene may serve other functions. The inconsistency between expression regulation of the genes and the presumed function calls for further investigations. Similarly, the two pyruvate kinase genes, *pykA* and *pykF*, were nonregulated and down-regulated, respectively, in acetate. This result suggests that *pykA* serves a different role than *pykF*, which plays the glycolytic role. In addition, many unknown or poorly characterized open reading frames were differentially regulated in acetate compared with glucose cultures. Those data are expected to provide important information to predict their functions in the future.

Acetate Shock—The effect of acetate shock induced by high concentrations of sodium acetate (100 mM) was previously studied with membrane arrays (13). Those experiments aimed to study the physiological response of *E. coli* to a sudden addition of a high concentration of acetate during growth in a glucose minimal medium. In contrast, we monitored the transcript profile of *E. coli* during balanced growth in an acetate medium with a nontoxic concentration (42 mM). As expected, the results from these experiments were not the same. Although there were some common phenomena, such as down-regulation of ribosomal proteins and up-regulation of *katE*, *dhnA*, *talA*, and *pflB*, most transcript profiles in this experiment were different from the acetate shock experiment. In particular, the effect of σ^S that was significant in acetate shock was not observed in acetate culture.

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