August 31, 2005

# A Global Regulatory Role of Gluconeogenic Genes in Escherichia coli Revealed by Transcriptome Network Analysis

Katy C. Kao, *University of California, Los Angeles*
Linh M. Tran, *University of California, Los Angeles*
James C. Liao, *University of California, Los Angeles*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 43, pp. 36079–36087, October 28, 2005


# A Global Regulatory Role of Gluconeogenic Genes in *Escherichia coli* Revealed by Transcriptome Network Analysis*[S]

Received for publication, July 27, 2005 Published, JBC Papers in Press, August 31, 2005, DOI 10.1074/jbc.M508202200

**Katy C. Kao, Linh M. Tran, and James C. Liao**[1]

*From the Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, California 90095*

**In bacterial adaptation to the dynamic environment, metabolic genes are typically thought to be the executors, whereas global transcription regulators are regarded as the decision makers. Although the feedback from metabolic consequence is believed to be important, much less is understood. This work demonstrates that the gluconeogenic genes in *Escherichia coli*, *ppsA*, *sfcA*, and *maeB*, provide a feedback loop to the global regulator, cAMP receptor protein (CRP), in carbon source transition. Disruption of one of the gluconeogenic pathways has no phenotype in balanced growth, but causes a significant delay in the diauxic transition from glucose to acetate. To investigate the underlying mechanism, we measured the transcriptome profiles during the transition using DNA microarray, and network component analysis was employed to obtain the transcription factor activities. Results showed that one of the global regulators, CRP, was insufficiently activated during the transition in the *ppsA* deletion mutant. Indeed, addition of cAMP partially rescued the delay in transition. These results suggest that the gluconeogenic flux to phosphoenolpyruvate is important for full activation of adenylate cyclase through the phosphorylated enzyme IIA$^{glu}$ of the phosphotransferase system. Reduction of this flux causes insufficient activation of CRP and a global metabolic deficiency, which exemplifies a significant feedback interaction from metabolism to the a global regulatory system.**

Bacteria adapt to environmental changes through various signaling pathways and alter the expression of metabolic genes to meet the growth requirements in the new conditions. In this paradigm, regulatory genes, particularly global regulatory genes, are the decision makers that provide unidirectional regulation to the metabolic genes. The feedback from metabolic consequence is much less understood, despite its presumed significance. This work demonstrates an example of such a feedback loop from metabolism to global regulation.

When *Escherichia coli* is grown on excess glucose, acetate is produced and excreted as excess carbon (1), the excreted acetate can be consumed as carbon source when glucose level drops (2, 3). During the metabolic switch from glucose to acetate, *E. coli* induces genes involved in acetate uptake, the glyoxylate shunt, the TCA cycle, as well as the gluconeogenic genes *pckA* and *ppsA* in different time scales (4). The genes *pckA* and *ppsA* belong to two parallel pathways for the gluconeogenic conversion of the TCA cycle intermediates to phosphoenolpyruvate (PEP)[2] with the malic enzymes (*sfcA* and *maeB*) and *ppsA* forming one path for the conversion of malate to PEP, and *pckA* forming the other for the conversion of oxaloacetate to PEP (Fig. 1).

The switch from glucose to acetate metabolism involves several regulators, such as CRP and IclR. In the absence of glucose, CRP is activated by cAMP, which is produced by adenylate cyclase. The activated cAMP-CRP adduct positively regulates a large number of uptake and metabolic genes to prepare for less favorable carbon sources. The induction of the glyoxylate shunt genes in acetate is mediated directly by IclR and IHF (5). The ligand for IclR activation has not been confirmed. Although phosphoenolpyruvate has been suggested as a ligand for the deactivation of IclR (6), another group was not able to show the same result (7).

This study originated from an interesting microarray result comparing *E. coli* transcript levels in acetate *versus* glucose minimal media during balanced growth (8). The gluconeogenic gene, *ppsA*, which is known to be non-essential for growth in acetate, is one of the most up-regulated genes in acetate compared with glucose growth. Indeed, disruption of *ppsA* has no observable phenotype in acetate or glucose minimal media under balanced growth. Then, why does *E. coli* need to induce a non-essential gene to such a high level?

In this work, we first found that *ppsA* is important during the transition state between glucose and acetate, although not essential in balanced growths in either carbon source. The *ppsA* mutant caused a significant lag during the diauxic transition. Through whole genome transcriptome profiling and network component analysis (NCA) (9, 10), we identified that CRP is insufficiently activated without *ppsA* during glucose to acetate transition. Disruption of other pathway genes to PEP also showed the same phenotype, suggesting that the PEP supply during the transition state is the regulatory return loop from metabolism to modulate cAMP production. Insufficient supply of PEP reduces the phosphorylated *crr* gene product, EIIA$^{glu}$, which is the activator of adenylate cyclase. This result demonstrates that the metabolic feedback of the gluconeogenic genes is important in determining transition time regulation, which is closely linked to bacterial survival in a dynamic environment.

## MATERIALS AND METHODS

*Strains and Culture Conditions—E. coli* BW25113 (F-(araD-araB) lacZ4787 lacIp-4000 LAM-rph-1 (rhaD-rhaB) hsdR514) was obtained from the Yale *E. coli* stock center and is used for all physiological studies and for monitoring transcript levels unless specified otherwise. Cells were grown in M9 minimum media (11) containing either 0.5% glucose or 0.25% acetate. In the abrupt carbon source transition experiments, cells were grown to mid-log phase ($A_{600}$ of 0.5–0.6), chilled quickly in

* This work was supported in part by National Science Foundation ITR program Grant CCF-0326605 and the UCLA IGERT Bioinformatics program supported by National Science Foundation Grant DGE9987641. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables A1 and A2.

[1] To whom correspondence should be addressed: UCLA, 420 Westwood Plaza, Los Angeles, CA 90095. Tel.: 310-825-1656; Fax: 310-206-4107; E-mail: liaoj@ucla.edu.

[2] The abbreviations used are: PEP, phosphoenolpyruvate; CRP, cAMP receptor protein; NCA, network component analysis; TFA, transcription factor activities; CS, control strength.

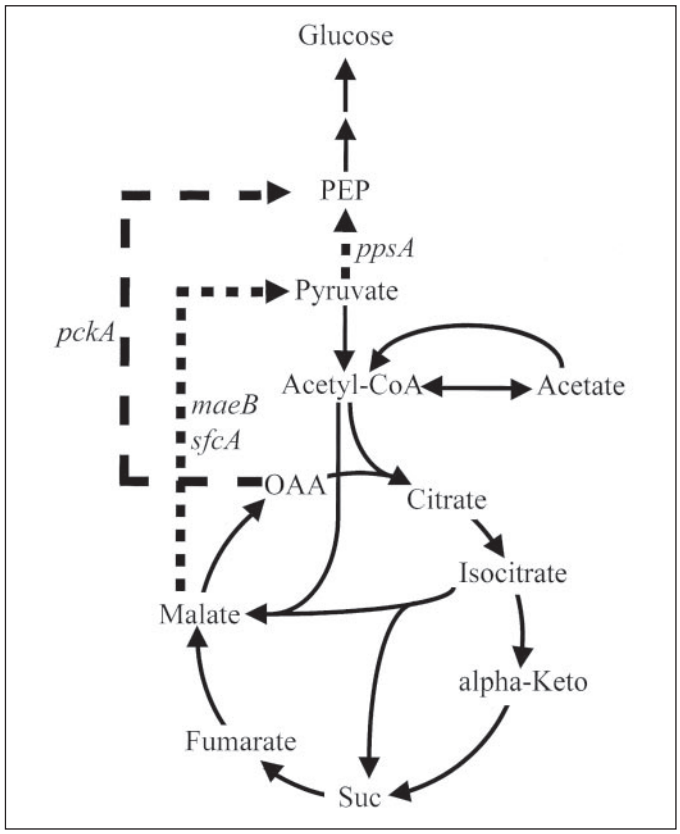


FIGURE 1. **Alternative pathways from the TCA cycle to PEP.** *Dashed line*, conversion of oxaloacetate (OAA) to PEP via *pckA*. *Dotted line*, conversion of malate to PEP via the malic enzymes (*maeB* and *sfcA*) and *ppsA*.

ethanol/dry ice bath, harvested by centrifugation at 8,000 × *g* at 4 °C, then rinsed once with 4 °C 0.25% M9 acetate. The cells were reinocuated into 0.25% M9 acetate media pre-warmed to 37 °C with starting $A_{600}$ of ~0.2. For gene expression studies, a portion of the cells was harvested as reference samples immersed in RNAlater (Qiagen, Valencia, CA), and the remaining cells were reinoculated into 0.25% M9 acetate media pre-warmed to 37 °C with starting $A_{600}$ of ~0.2. Time course samples were collected at specified times by quickly chilling in a ethanol/dry ice bath and harvested by centrifugation at 8,000 × *g* at 4 °C. All samples for time-dependent gene expression studies were kept in RNAlater (Qiagen, Valencia, CA) at −80 °C for RNA purification at a later time. For cAMP addition experiments, 10 mM cAMP (Sigma) was added to the pre-warmed 0.25% M9 acetate cultures.

*Deletion Mutation and Plasmid Construction*—Each gene was disrupted using the PCR-based method developed by Datsenko and Wanner (12). Primer sequences for deletion and verification are described previously (13). Briefly, the chloramphenicol resistance gene was PCR amplified from pKD3 using primers containing sequences homologous to the target gene locus. The linear PCR product was transformed into BW25113 containing the λ helper plasmid pKD46 to disrupt the target gene. The chloramphenicol cassette was removed by transforming pCP20 into the resulting deletion mutant strain and selected for transformants with loss of chloramphenicol resistance. Multiple deletion mutants were constructed sequentially.

The plasmid pTB108 was constructed from pJF118EH (14) by replacing part of the lactose repressor gene, *lacI*, and the P*tac* promoter with the arabinose repressor (*araC*) coding sequence including its promoter and the arabinose promoter (P*araBAD*) from pBAD (Invitrogen) and fusing the P*araBAD* to $gfp_{mut3.1}$, which was amplified from $pGFP_{mut3.1}$ (15) by PCR. The $gfp_{mut3.1}$ open reading frame is flanked by the unique restriction sites, XbaI on the 5′ end and HindIII on the 3′ end. Each gene was amplified by PCR using the primers listed in TABLE ONE and inserted into pTB108 between XbaI and HindIII to generate pTB108::*ppsA*, pTB108::*pckA*, and pTB108::*maeB*.

*DNA Microarray Design, Procedures, and Data Analysis*—The array design, RNA purification, and hybridization procedures were described previously (13). Each slide contained two spots of the same probe, each sample was hybridized to two slides, and each time course experiment was replicated up to three times. The resulting images were scanned using a VersArray ChipReader High Resolution (5 um) (Bio-Rad) scanner at two excitation wavelengths (532 nm for Cy3 and 635 nm for Cy5). The two scanned images were analyzed using the image analysis software, Imagene (Biodiscovery, Marina Del Rey, CA). The median intensities for signal and background were obtained from the software and used for downstream analysis. The software package, lcDNA (16), developed by our laboratory based on methods described in Tseng *et al.* (17), was used to normalize the data and calculate the 97.5 and 2.5 quantiles of gene expression, and to assess the significance of expression for each gene. The resulting averaged value of the 97.5 and 2.5 quantiles for each normalized log ratio was used for subsequent analysis.

*Transcription Factor Activity Analysis*—NCA was used to reconstruct the transcription factor activities (TFA) and control strengths (CS) from DNA microarray data (4, 9, 10). Briefly, the gene expression is modeled by power law (18, 19),

$$[\mathbf{E}] = [\mathbf{CS}] \cdot [\mathbf{TFA}] \qquad \text{(Eq. 1)}$$

where [**E**] is the matrix of log (base 10) gene expression ratio, [**TFA**] is the log ratio of transcription factor activities, and [**CS**] is the matrix of control strengths. The NCA decomposition of [**E**] into [**CS**] and [**TFA**] is based on the topology of [**CS**], which is the existence of connection between transcription factors and their regulated genes. These connectivity relationships in *E. coli* were obtained from RegulonDB (20) with additional modifications obtained from literature and experiments (the connectivity information used can be found at www.seas.ucla.edu/~liaoj/), which includes 834 genes regulated by 124 transcription factors. However, not all the genes showed differential transcript abundance, and thus the transcription factors whose regulons were deemed unperturbed were eliminated from the analysis. The final NCA network that satisfied the first 2 criteria was composed of 302 genes regulated by 34 transcription factors. The network was analyzed independently for different datasets. Because we scaled the solution sets such that absolute averages of the control strengths were equal to 1 (4, 9), the TFAs of the same transcription factor were in the same scale and comparable. NCA software are available from the web site (www.seas.ucla.edu/~liaoj/).

## RESULTS

### *ppsA Determines the Diauxic Transition Time*

A *ppsA* deletion mutant in BW25113 was generated using a PCR-based method developed by *Datsenko et al.* (12). We first compared the growth kinetics of the *ppsA* mutant strain with its otherwise isogenic parent in glucose and acetate minimum media. No growth rate difference was detected between the two strains (Fig. 2, *a* and *b*). However, if both glucose and acetate were present simultaneously, we observed a significantly longer diauxic lag in the *ppsA* strain than the parent (Fig. 2*c*). Thus, *ppsA* may be involved in the transition time regulation. To focus on the carbon source transition, we designed an abrupt carbon source transition experiment as described under "Materials and Methods." Such experiments yield better reproducible transition time (Fig. 2*d*).

**TABLE ONE**

**Primers used for cloning**

Underlined sequences represent restriction sites.

| Primer | Sequence (5′ to 3′) |
|---|---|
| ppsA-for-XbaI | aaaaaaatctagaatgtccaacaatggctcgtc |
| ppsA-rev-HindIII | aaaaaaaaagctttttatttcttcagttcagcca |
| pckA-for-XbaI | aaaaaaaaagctttttatttcttcagttcagcca |
| pckA-rev-HindIII | aaaaaaaaagctttttacagtttcggaccagccg |
| maeB-for-NcoI | aaaaaaaccatggattaaagaggagaaatctagaatggatgaccagttaaaacaa |
| maeB-rev-HindIII | aaaaaaaaagctttttacagcggttgggtttgcg |

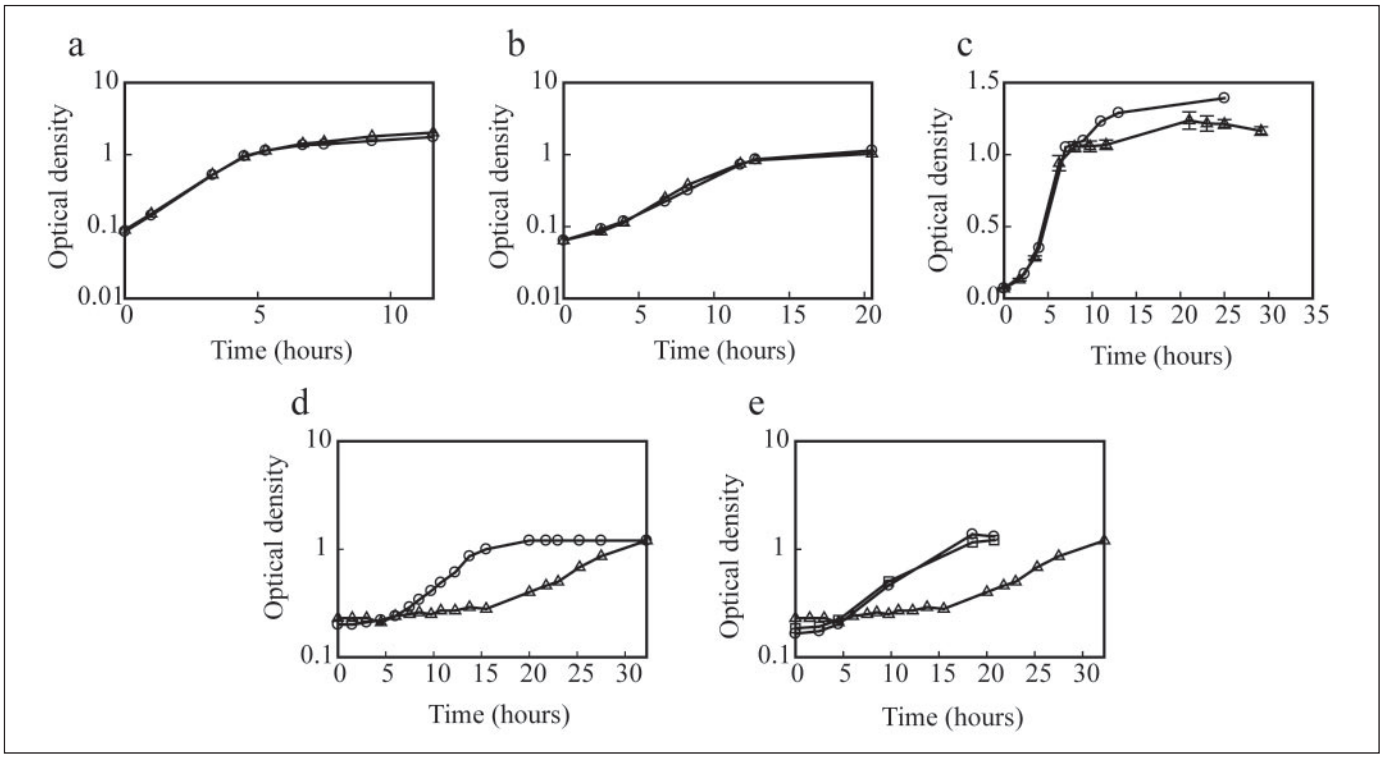


FIGURE 2. **ppsA growth phenotype: wild-type (*circle*) and *ppsA* (*triangle*).** *a*, glucose-balanced growth; *b*, acetate-balanced growth; *c*, glucose to acetate diauxie in 0.15% glucose + 0.09% acetate (*circle*, representative wild-type growth curve; *triangle*, *ppsA* growth average over five independent cultures, *error bars* represent standard deviation); *d*, glucose to acetate transition; and *e*, rescue of *ppsA* phenotype by *pckA* overexpression (pTB108::*pckA*).

To eliminate the possibility that this is a strain-dependent phenomenon, we also deleted the *ppsA* gene in another *E. coli* K-12 strain, MC4100, and found that the *ppsA* mutant strain in MC4100 also exhibited a longer growth lag during the transient state between glucose and acetate (data not shown). Note that the *ppsA* mutant was constructed by removing the whole open reading frame of the *ppsA* gene, thus the possibility of a revertant is practically eliminated. Another explanation for why the *ppsA* mutant strain was able to resume growth after a long lag is the possibility that it acquired a second-site suppressor mutation that allowed it to exhibit the wild-type phenotype. Therefore, we took samples of the *ppsA* mutant strain after the glucose to acetate transition experiment and conducted a second round of carbon source transition. Again, the *ppsA* mutant strain exhibits the same phenotype after a second passage, suggesting the phenomenon is not a result of a second-site mutation. To confirm that the decreased ability of the *ppsA* mutant strain to adapt to acetate is because of lack of a functional *ppsA* gene and not an artifact of the strain construction, a vector was constructed to express a functional copy the *ppsA* gene in *trans* from a plasmid (pTB108::*ppsA*). The presence of pTB108::*ppsA* in the *ppsA* mutant strain was able to complement the longer growth lag exhibited by the *ppsA* mutant strain (data not shown).

### *The Transition State Dependence of pckA Is Strain Dependent*

Because *pckA* and *ppsA* form parallel pathways from the TCA cycle to PEP, we tested whether the *pckA* deletion mutation caused a similar phenotype as the *ppsA* mutant during the glucose to acetate transition. The *pckA* deletion mutant exhibited no growth phenotype during balanced growth in either glucose or acetate. However, unlike *ppsA*, the *pckA* deletion mutant exhibited no significant growth phenotype from wild-type during the glucose to acetate transition (Fig. 3). The dependence of *pckA* appears to be strain dependent as a prolonged growth lag was observed in a *pckA* deletion mutant in an MC4100 strain during the carbon source transition (Fig. 3*d*). Note that it has been suggested that *pckA* is the main gluconeogenic gene for growth in acetate metabolism (1). Apparently, the relative importance of the *pckA* and *ppsA* pathways are strain-dependent.

### *Global Gene Expression Pattern*

*Similarity between the Wild-type and the ppsA Mutant*—To probe the transcriptional regulations involved in the *ppsA* mutant phenotype during the transition from glucose to acetate, the time course expression profiles of BW25113 wild-type and *pps* strains were monitored

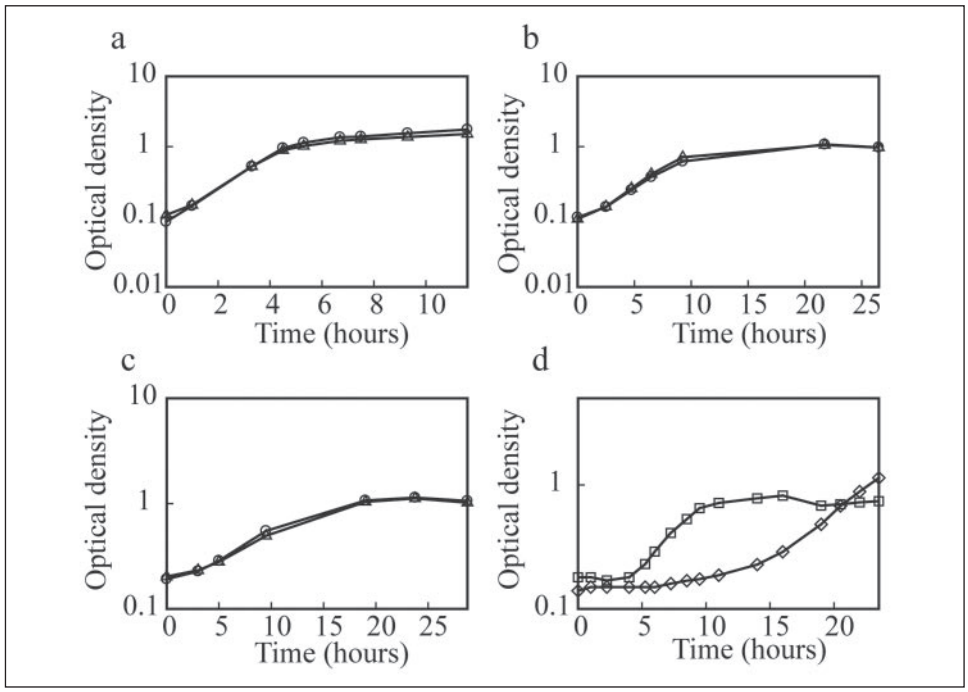


FIGURE 3. **pckA growth phenotype BW25113 wild-type (*circle*), BW25113 *pckA* (*triangle*), MC4100 wild-type (*square*), and MC4100 *pckA* (*diamond*).** *a*, glucose-balanced growth; *b*, acetate-balanced growth; *c*, BW25113 glucose to acetate transition; *d*, MC4100 glucose to acetate transition.

using cDNA microarrays spotted with ~96% of open reading frames in the *E. coli* genome. The microarray chip design and experimental procedures are described under "Materials and Methods." Time course experiments were performed for each strain where samples were harvested at times 5, 15, 30, 60, 120, 180, 240, 300, and 360 min after transition. Each time course sample was cohybridized with a reference sample, which was harvested immediately prior to transition into acetate media (designated 0th time sample). Each sample was hybridized to duplicate slides. Each experiment was repeated up to three times. The expression ratio, as well as confidence intervals of expression of the time course sample over reference sample, for each gene was determined using the software package lcDNA (16, 17).

We previously reported the transcriptional gene expression dynamics for the same wild-type strain (4). In the wild-type strain, a growth lag of ~2–3 h is observed during the transient state between glucose and acetate. During this growth lag, transcriptional regulation was highly active with 331 genes induced and 437 genes repressed by at least 2-fold in at least 2 of the 4 time points within the first hour. Even though the *ppsA* strain exhibited a much longer growth lag, highly active transcriptional regulation was observed in the *ppsA* strain with a total of 362 gene transcripts increased at least 2-fold and 392 gene transcript decreased at least 2-fold in at least 2 of the 4 time points within the first hour.

Several genes known to be involved in acetate metabolism, such as the glyoxylate shunt enzymes (*aceBAK*) (21), *acs* (2), *pckA* (8), and *glcB* (24) showed increased transcript levels within 1–2 h of transition for both strains. In fact, *acs* and *pckA* were highly induced (>10-fold) within the first 5 min into the transient state in both strains. We reported earlier (4) that several of the TCA cycle genes were initially down-regulated in the wild-type strain within the first hour of transition. The same trend in gene expression were also observed in the *ppsA* strain. As a control, we performed a similar transition experiment from glucose to glucose instead of glucose to acetate. Time points were taken at 5, 15, and 30 min after the glucose to glucose transition and gene expression profiles were measured using DNA microarrays. These TCA cycle genes were not affected in the sham transition experiment, verifying that the down-regulation of these genes were not a result of the transition treatment. Roughly 110 genes were perturbed total in the sham transition experiment. Surprisingly, several genes involved in the translational machinery (*e.g. rplKA* and *rplCDW*) showed increased expression, and the stress-related genes *dps* showed decreased transcript levels during the sham experiment, suggesting the transitional treatment did not cause significant stress to the cell.

Comparison between the post-transition ($t > 0$) and pre-transition samples ($t = 0$) revealed an overall down-regulation of the translational machinery (*e.g. rplNXFRO*, *rpsE*, *rpmOJ*, and *rplKA*). Both the wild-type and the *ppsA* strains showed reduced transcript levels in the amino acid biosynthesis pathway genes (*e.g. thrAC*, *hisIHDC*), as well as the nucleotide biosynthesis genes (*e.g. purA*, *purB*, *purF*, *guaBA*), during the initial stages of transition from glucose to acetate. Exceptions include the cysteine biosynthesis genes (*e.g. cysE*, *cysM*, *cysK*). On the contrary, several carbohydrate transport and metabolism genes (*e.g. rbsABD*, *malGF*) were induced in both strains, likely as a means to scavenge other carbon sources in the absence of glucose. Among the genes that were up-regulated in both strains within the initial hour of transition, roughly 45% code for hypothetical proteins or proteins of putative functions. Transition from glucose to acetate resulted in the induction of several genes known to be involved in acid resistance, such as *xasA*, *yhiE*, and *hdeAB*.

The recently identified acetate permease, *yjcG*, which is cotranscribed with the acetyl-coA synthase gene (*acs*) (25) is induced in both strains during the carbon source transition. Two proteins, CobB and Pat, were recently identified in *Salmonella enterica* to regulate *acs* activity post-translationally (26, 27). The CobB protein is the deacetylase that activates Acs activity, whereas Pat deactivates the ATP-dependent adenylation of acetate by acetylating Acs. In the *E. coli* glucose to acetate transition, the transcript level of the Pat homolog, *b2584*, was increased, whereas the transcript of CobB was not differentially regulated during the glucose to acetate transition.

*Difference between the Wild-type and the ppsA Mutant*—From our wild-type gene expression data (4),[3] we found that the *ppsA* gene was

[3] The gene expression data has been submitted to Gene Expression Omnibus accession number GSE3250.

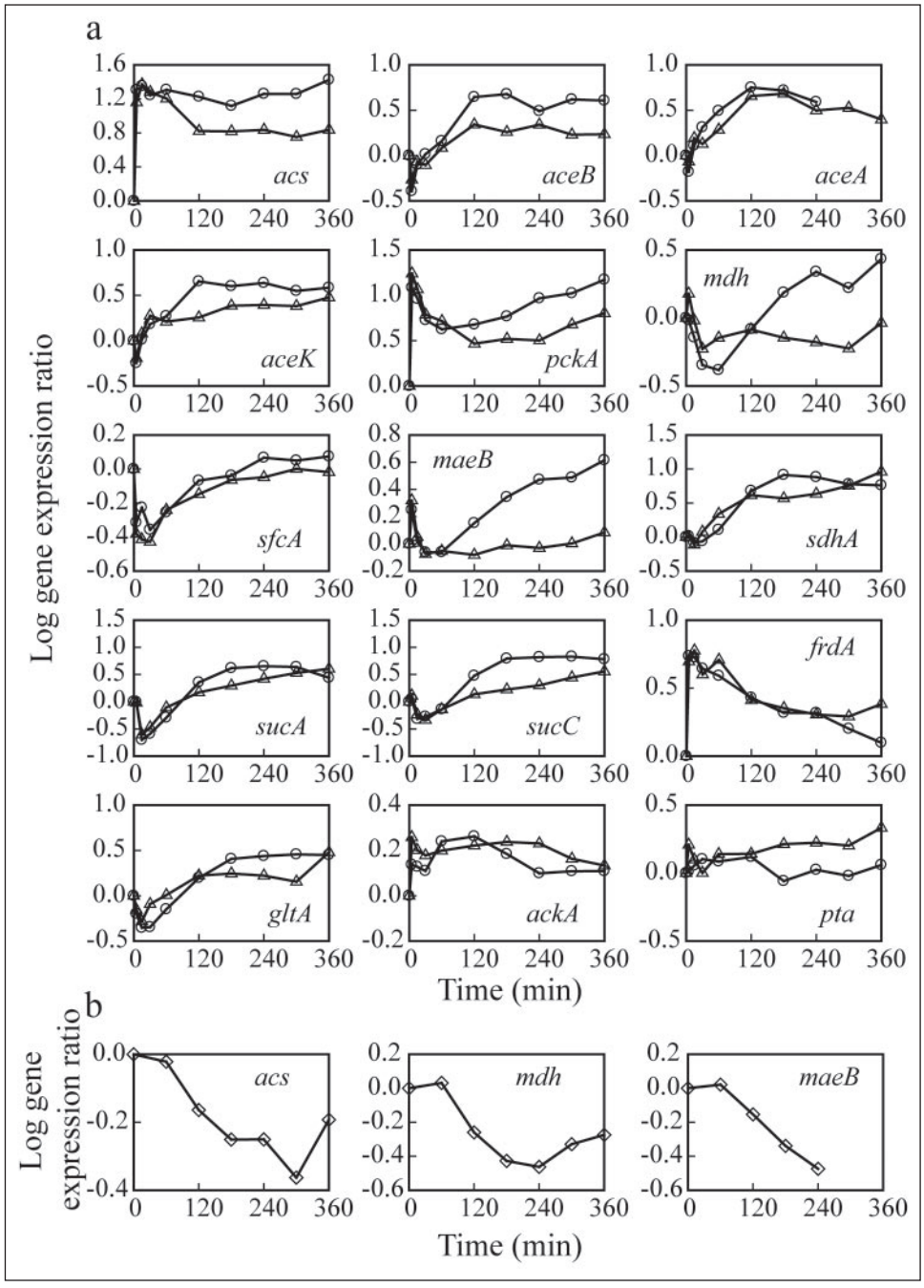


FIGURE 4. *a*, gene expression profiles of acetate metabolic genes; *circle*, wild-type; *triangle*, *ppsA*. *b*, relative gene expression of selected CRP-regulated genes in *ppsA versus* wild-type during glucose to acetate transition.

not induced until ~2–3 h after the cells were shifted to acetate. Thus, we would expect the effect of the *ppsA* mutation on overall gene expression to be minimal within the first hour after transition and increase as the cells respond to the lack of this gluconeogenic pathway. Indeed, in the first hour within the transient state, the expression profiles of *ppsA* and wild-type strains were very similar, with the Pearson correlation coefficients around 0.9. Between 2 and 6 h during the carbon source switch, the Pearson correlation coefficient between wild-type and *ppsA* strains decreases from 0.7 to 0.5. This decrease in correlation coefficient between the two strains is partly because of the growth related differences between them. For example, the transcript levels of genes involved in the translational machinery (*e.g. rplNXFRO*, *rpsE*, *rpmDJ*, *rplKA*), which were initially reduced in both strains, stayed reduced longer in the *ppsA* mutant strain. The same trend is observed in some genes involved in biosynthesis such as the threonine biosynthesis genes (*e.g. thrABC*).

Most TCA cycle genes were up-regulated within 2 h of transition for both strains (Fig. 4). However, one notable exception, the malate dehydrogenase gene, *mdh*, whose induction is observed within 4 h of transition in the wild-type strain but not until within 10 h of transition in the *ppsA* mutant strain. In addition, the gene expression of the NADP-linked malic enzyme (*maeB*) was significantly differentially regulated between the wild-type and *ppsA* strains (Fig. 4*a*).

The DNA-binding protein from starved cells, Dps, is a nucleoid protein involved in the survival of *E. coli* under various environmental conditions including long term stationary phase survival, oxidative stress, acidic and alkaline pH conditions (28). The gene expression of *dps* is regulated by the stationary $\sigma$ factor, OxyR, and IHF (29). During the

glucose to acetate transition, the *dps* transcript level is increased in both wild-type and *ppsA* mutant strains. However, the induction of *dps* transcription in the wild-type strain decreased as the cells resumed growth by time = 4 h after the carbon source transition, whereas it remained induced in the *ppsA* strain. This prolonged induction of the *dps* transcript level may be indicative of a prolonged stress in the *ppsA* mutant strain during the glucose to acetate transition.

### *Transcription Factor Activities Deduced Using NCA*

The genes involved in acetate metabolism are under the control of various transcriptional regulators, such as CRP, FruR (Cra), IclR, FIS, IHF, RpoS, FNR, and ArcA. Because these regulators respond to different signals, it is difficult to decipher which ones malfunctioned because of *ppsA* deletion. Thus, NCA (9, 10) was used to determine the activities of these transcription factors in the wild-type and *ppsA* mutant strains during the glucose to acetate transition. NCA combines microarray data with regulator-promoter binding data from RegulonDB (20) to deduce the regulatory activity of transcription factors of interest. It was able to deconvolute the relative contributions of each regulator from a complex interaction network. In addition to the protein regulators that directly bind to DNA, a small molecule, guanosine tetraphosphate (ppGpp), is known to participate in the response to stringent conditions and growth rate changes (30). The genes affected by ppGpp, directly or indirectly, typically are involved in amino acid biosynthesis, stable RNA synthesis (31), as well as the stationary phase $\sigma$ factor (32, 33). It has been shown that ppGpp is increased during nutritional downshift (34), and thus it is likely that the ppGpp level is increased during the transition from glucose to acetate. To account for the regulatory effects because of ppGpp during the glucose to acetate transition, we included in NCA a "stringent factor" that accounts for the direct and indirect effects of gene expression caused by growth rate difference. The "connectivity" between the stringent factor and genes was determined in a separate microarray experiment by comparing transcriptome before and after the addition of serine hydroxymate.[4]

A total of 34 transcription factors (including the stringent factor) were analyzed based on two sets of micorarray experiments with each set containing 10 time points of data lumped from 2 to 6 slides per time point. The key results of transcription factor activities are shown in Fig. 5*a*, and the complete results are given in the supplemental data. The activity of CRP, which requires binding of cAMP for activation, peaked within the first hour in both wild-type and *ppsA* mutant strains after the glucose to acetate transition. However, after the first hour, the activity of CRP in the *ppsA* strain dropped to a level below that of the wild-type strain. The activity of the stringent factor was high during the initial transition from glucose to acetate in the wild-type strain. However, it decreased to the reference level (mid-exponential growth on glucose) after ~4–5 h in the wild-type strain, but remained appreciably active in the *ppsA* strain. The stationary phase $\sigma$ factor, RpoS, whose level increases under starvation conditions (32, 35, 36), showed activity profiles corresponding to the growth difference between the two strains.

The nucleoid-associated proteins, FIS and IHF, regulate several genes involved in acetate metabolism. Because no effectors have been identified for FIS activation and it is primarily regulated transcriptionally (37), its activity corresponds to its transcript level. As shown in Fig. 5, *a* and *b*, FIS activity correlated with its transcript levels in both the wild-type and *ppsA* strains, confirming the results of NCA. The activity of FIS showed an initial reduction during the transition from glucose to acetate in both wild-type and *ppsA* strains. However, because the FIS level reaches maximum coming out of stationary phase (38), its activity increased in the wild-type strain as cells start to come out of growth lag, but not in the *ppsA* strain because of the longer growth lag. Activities of other transcription regulators were not readily explainable.

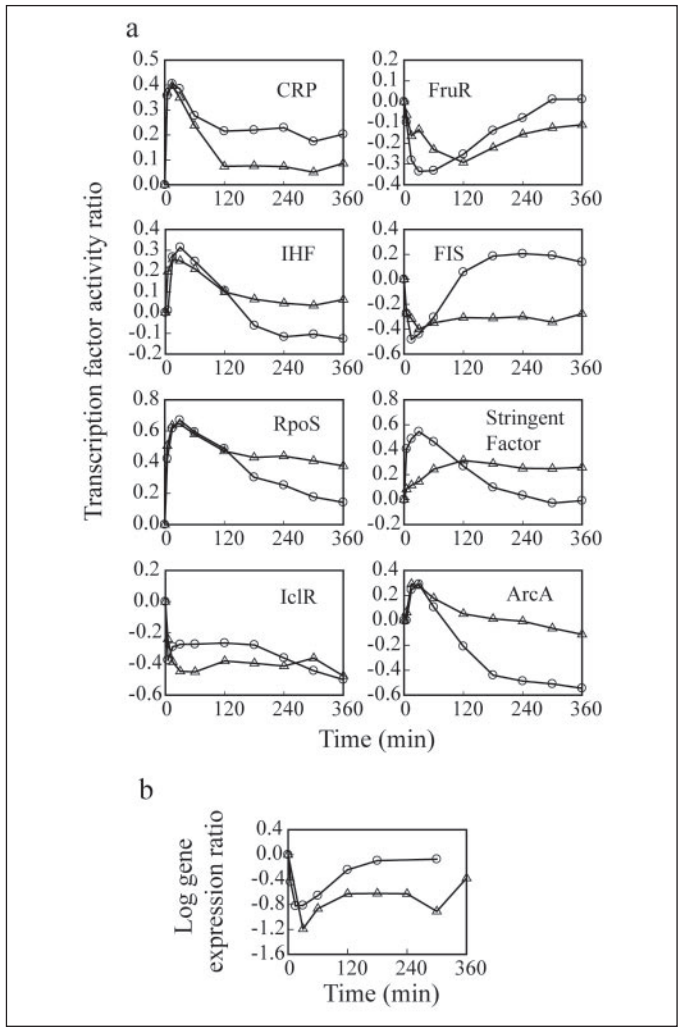


FIGURE 5. *a*, TFA for selected transcription factors. *b*, gene expression profile of *fis*. *Circle*, wild-type; *triangle*, *ppsA* mutant.

### *Regulatory Implications of ppsA Mutation*

Among the transcription factor activities that are affected by the *ppsA* mutation, CRP is the most interesting. *ppsA* catalyzes the reaction from pyruvate to phosphoenolpyruvate, which, besides providing precursors for biosynthesis, is also involved in the phosphoenolpyruvate-dependent sugar phosphotransferase system. It has been suggested that the ratio between phosphoenolpyruvate and pyruvate can affect the phosphorylation state of the $EIIA^{glu}$ protein in the phosphotransferase system in *E. coli* (39, 40). Because one of the factors controlling cAMP production in *E. coli* is the phosphorylation state of the $EIIA^{glu}$ protein, the absence of *ppsA* may cause an insufficient activation of CRP during the transient state. Several genes involved in acetate metabolism are known to be under the regulation of CRP-cAMP. The malate dehydrogenase gene has a putative CRP binding site and has been suggested to be regulated by CRP (41). The expression of *mdh* had a delayed induction in the *ppsA* mutant strain. The NADP-linked malic enzyme gene, *maeB*, whose gene expression was not induced within the first 6 h of

[4] L. Jarboe, manuscript in preparation.

transition in the *ppsA* strain, was recently identified as a potential member of the CRP regulon (42). The primary acetate uptake gene, *acs*, although showed similar expression profiles in both wild-type and *ppsA* strains, had a higher expression in the wild-type strain than the *ppsA* strain after 2 h into the transient state. CRP is an important activator for the *acs* gene (43, 44). These differences in relative gene expressions were confirmed from the direct comparison experiments, where the gene expression of the *ppsA* mutant was directly compared against the wild-type strain at times = 1–6 h after transition from glucose to acetate (see Fig. 4*b*). The results obtained suggest that CRP may be insufficiently activated in the *ppsA* strain compared with the wild-type strain.

### *cAMP Rescue of ppsA Phenotype*

Because cAMP is the inducer for CRP activation, we added 10 mM cAMP to both wild-type and *ppsA* cultures during the glucose to acetate transition to determine whether the addition of cAMP helps to rescue the *ppsA* phenotype. Results are shown in Fig. 6*a*. The addition of cAMP to the wild-type culture resulted in a slightly longer growth lag, possibly because of metabolic imbalance because of the overactivation of CRP. However, the addition of cAMP to the *ppsA* strain resulted in a shorter growth lag during the transient state. This rescue, although incomplete, is indicative of a regulatory effect of the *ppsA* mutation during the glucose to acetate transition. The partial rescue showed that the PEP supply is not only needed for the activation of CRP, but also important for biosynthetic precursors.

### *Effect of cAMP Addition to Gene Expression*

To further support the hypothesis that the partial rescue by cAMP addition in the *ppsA* strain is because of CRP activation, time-dependent gene expression profiles were obtained during the glucose to acetate transition with or without the addition of cAMP. Samples were harvested at the same time intervals as before.

If the partial rescue by cAMP is via the activation of CRP, then we would expect to see an earlier induction of the CRP-regulated genes in the *ppsA* strain that showed a lower up-regulation or slower induction. The TCA cycle gene, *mdh*, that showed a much slower induction in the *ppsA* strain was induced by ~2-fold within 5 h of transition with the addition of cAMP. The NADP-dependent malic enzyme gene, *maeB*, which was induced in the wild-type strain within 3 h of transition but not in the *ppsA* strain, showed increased transcript levels by ~1.7-fold within 5 h into the transient state in the *ppsA* strain with the addition of cAMP. The addition of cAMP also increased the expression of *acs* in the *ppsA* mutant strain during the glucose to acetate transition (Fig. 6*b*).

### *Rescue by Alternate Pathway*

It has previously been shown that PckA and the malic enzymes-PpsA are the only pathways providing PEP during acetate growth (13). During steady-state acetate growth, the *pckA* pathway provides sufficient carbon flux to PEP in the *ppsA* mutant. However, during the transient state between glucose and acetate, the *pckA* pathway may not be sufficiently compensating for the *ppsA* strain. Thus, to determine whether the *ppsA* mutant phenotype was because of insufficient gluconeogenic flux to PEP, we overexpressed PckA in the *ppsA* strain. The overexpression of the alternate pathway was sufficient to rescue the *ppsA* phenotype during the glucose to acetate transition (see Fig. 2*e*). This result proved that the longer adaptation period in the *ppsA* strain was because of insufficient carbon flux to PEP.

To further support that the *ppsA* phenotype resulted from insufficient gluconeogenic flux to biosynthetic precursors, 0.02% casamino acid was added to the bacterial cultures during the glucose to acetate transition. The addition of casamino acids reduced the *ppsA* growth lag to that of the wild-type strain but did not improve the wild-type growth during the carbon source transition (data not shown).

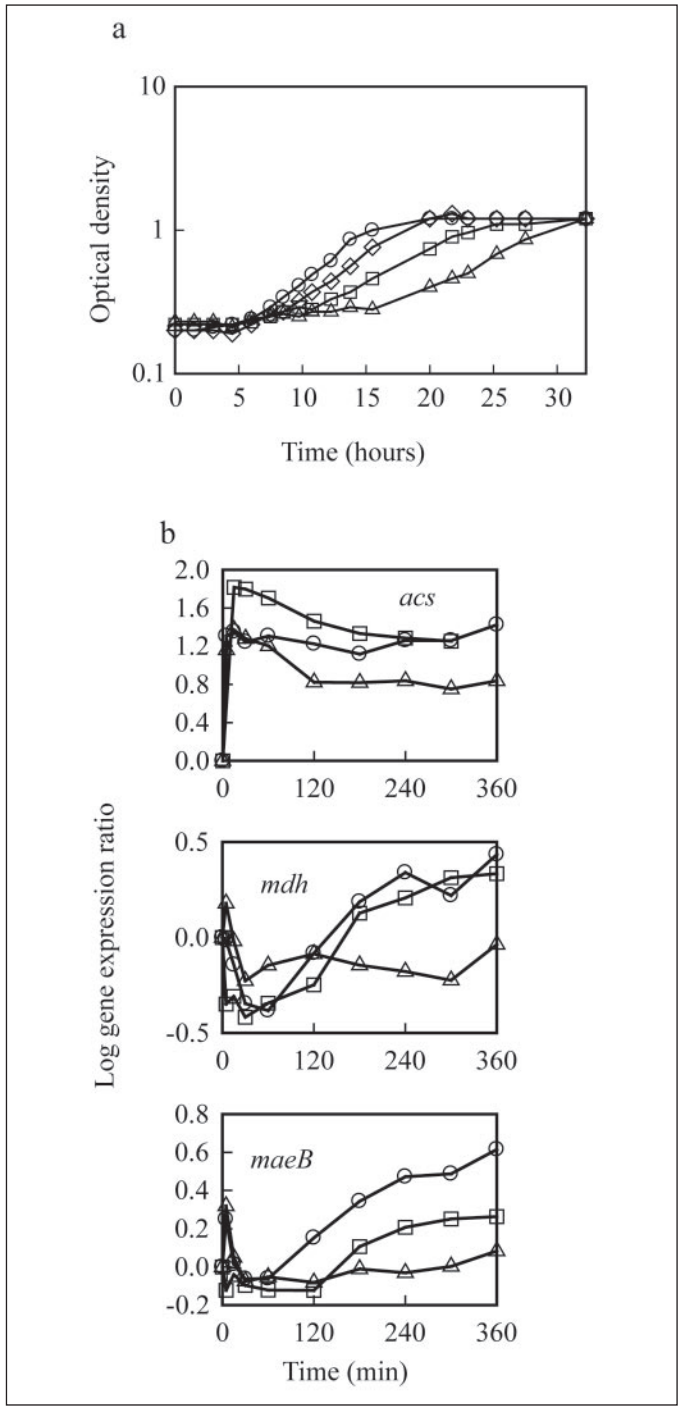


FIGURE 6. **cAMP rescue.** *a*, partial rescue of *ppsA* phenotype during glucose to acetate transition; *b*, effect on key members of CRP regulon by addition of cAMP to the *ppsA* strain: wild-type (*circle*), wild-type with 10 mM cAMP addition (*diamond*), *ppsA* (*triangle*), and *ppsA* with 10 mM cAMP addition (*square*).

### *Malic Enzymes Are Also Important in the Transition Phase*

Because the malic enzymes (SfcA and MaeB) and PpsA form one of the metabolic pathways from malate to PEP, it is likely that the malic enzymes also are important during the transition phase. Both malic enzyme genes, *sfcA* and *maeB*, were found to have induced gene expres-

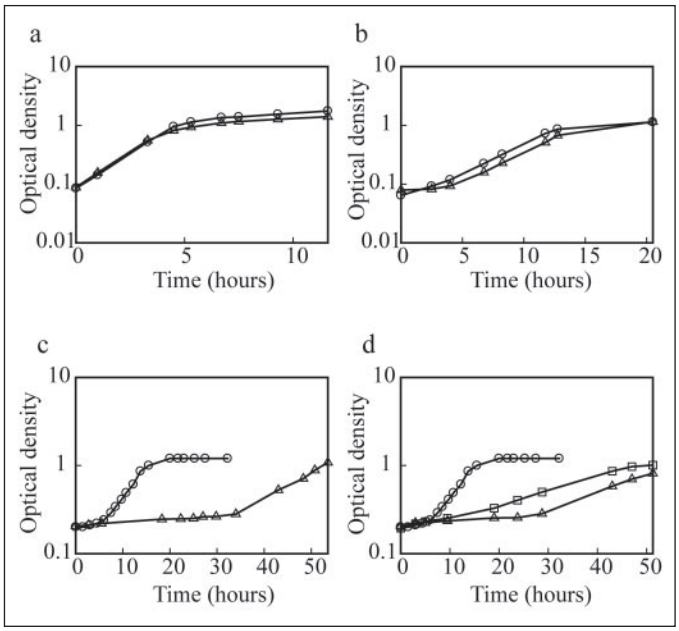


FIGURE 7. **maeB sfcA growth phenotype: wild-type (*circle*), *maeB sfcA* (*triangle*), *maeB sfcA* (*square*) with 10 mM cAMP addition.** *a*, glucose-balanced growth; *b*, acetate-balanced growth; *c*, glucose to acetate transition; *d*, partial rescue of *maeB sfcA* growth phenotype by 10 mM cAMP addition during glucose to acetate transition.

sion during acetate metabolism (13), indicative of a physiological role on this carbon source. To investigate this possibility, mutant strains of either *sfcA* or *maeB* and a *sfcA maeB* double mutant were generated as described above. Individual mutations of either *sfcA* or *maeB* resulted in no significant growth phenotype during balanced growth in either glucose or acetate metabolism or during the transition from glucose to acetate, suggesting that the activity of either one of the malic enzymes can sufficiently catalyze the malate to pyruvate conversion. However, the *sfcA maeB* double mutant showed a much more severe lag phase during the transient state between glucose and acetate while exhibiting no growth rate differences from the wild-type strain during balanced growth in either glucose or acetate alone (Fig. 7). Again, this phenotype was shown to be independent of second-site suppressor mutation occurring during the long lag phase (data not shown). Furthermore, overexpression of *maeB* was able to complement the *sfcA maeB* phenotype during glucose to acetate transition, confirming that the long lag phase was indeed a result of flux deficiency (data not shown). However, unlike *ppsA* mutation, overexpression of *pckA* did not fully rescue the *sfcA maeB* double mutation. In fact, overexpression of both *mdh* and *pckA* were not able to fully rescue the *sfcA maeB* mutant phenotype, indicating that the malic enzymes play additional roles other than carbon flux to PEP.

The insufficient flux to PEP caused by the *sfcA maeB* mutant during glucose to acetate transition should have regulatory implications similar to the *ppsA* mutant. Addition of exogenous cAMP to the *sfcA maeB* double mutant during the carbon source transition resulted in a partial reduction in the growth lag, indicating that CRP is also insufficiently activated in the *sfcA maeB* strain (see Fig. 7*d*).

## DISCUSSION

In *E. coli*, two gluconeogenic pathways provide PEP from the TCA cycle, PckA, and the malic enzymes-PpsA pathways. PckA is thought to be the enzyme providing gluconeogenic flux during acetate metabolism (1). However, both *pckA* and *ppsA* genes are the most highly expressed on acetate-balanced growth compared with those in glucose-balanced growth (4, 13). In fact, deletion of either gene shows no phenotype during balanced growth in either glucose or acetate minimal media. Then, what is the role of such a highly induced non-essential gene? This answer was found during a carbon source transition between glucose and acetate: the deletion mutant of *ppsA* (or *sfcA maeB* double mutant) exhibited a substantially longer transition lag. This result demonstrates that gene function could not simply be defined during balanced growth: dynamic environmental changes are important.

Transcriptome analysis and NCA further point to the global regulator, CRP, as a key reason for the long lag. Indeed, addition of cAMP in the medium partially rescued the long transitional delay in both the *ppsA* mutant and the *sfcA maeB* double mutant. The regulatory effect caused by the *ppsA* mutation is likely mediated through the metabolic intermediate, PEP. The PEP/pyruvate ratio has been shown to affect the phosphorylation state of the $EIIA^{glc}$ (23, 39, 40), which is an important factor in the activation of adenylate cyclase activity. The rescue of the prolonged growth lag in the *ppsA* strain by overexpression of *pckA* suggest that the *ppsA* phenotype is a result of insufficient gluconeogenic flux to PEP.

It has been proposed that the presence of pyruvate during acetate metabolism altered the isocitrate dehydrogenase activity resulting in inhibited growth because of reduced carbon flux through the glyoxylate shunt (22). Thus, it is possible the loss of *ppsA* may have caused pyruvate accumulation during the glucose to acetate transition, causing the prolonged growth lag in the *ppsA* mutant strain. However, this did not appear to be the case, because the deletion of the pyruvate generating genes (*sfcA* and *maeB*) from the *ppsA* positive or negative (data not shown) strains did not alleviate the *ppsA* phenotype during the glucose to acetate transition. In fact, the *sfcA naeB* double mutant caused a even more pronounced transition phenotype (Fig. 7).

These results demonstrate an important idea in metabolic regulation: action caused by signaling molecules that reflect the external environment will feedback to dampen the signal. The CRP-mediated global regulation responds to the signaling molecule cAMP, which is thought to reflect the depletion of glucose. cAMP-activated CRP induces or represses a large number of genes that are necessary for alternative carbon source metabolism. In this scenario, the feedback pathway is not clear, because the alternative carbon metabolic pathways could not alter the external glucose concentration. Our work here demonstrated that the feedback pathway is through PEP (or the PEP/pyruvate ratio), which determines the level of phospho-$EIIA^{glc}$ and subsequently the activity of adenylate cyclase. This feedback allows the modulation of cAMP activity under a non-glucose carbon source.

*Acknowledgments—We thank Dr. Young L. Yang for helpful discussions and Dr. Thomas Buelter for construction of pTB108.*

## REFERENCES

1. Holms, H. (1996) *FEMS Microbiol. Rev.* **19,** 85–116
2. Shin, S., Song, S. G., Lee, D. S., Pan, J. G., and Park, C. (1997) *FEMS Microbiol. Lett.* **146,** 103–108
3. Wolfe, A. J. (2005) *Microbiol. Mol. Biol. Rev.* **69,** 12–50
4. Kao, K. C., Yang, Y. L., Boscolo, R., Sabatti, C., Roychowdhury, V., and Liao, J. C. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 641–646
5. Resnik, E., Pan, B., Ramani, N., Freundlich, M., and LaPorte, D. C. (1996) *J. Bacteriol.* **178,** 2715–2717
6. Cortay, J. C., Negre, D., Galinier, A., Duclos, B., Perriere, G., and Cozzone, A. J. (1991) *EMBO J.* **10,** 675–679
7. Yamamoto, K., and Ishihama, A. (2003) *Mol. Microbiol.* **47,** 183–194
8. Oh, M. K., and Liao, J. C. (2000) *Biotechnol. Prog.* **16,** 278–286
9. Liao, J. C., Boscolo, R., Yang, Y. L., Tran, L. M., Sabatti, C., and Roychowdhury, V. P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15522–15527
10. Tran, L. M., Brynildsen, M. P., Kao, K. C., Suen, J. K., and Liao, J. C. (2005) *Metab. Eng.*

**7,** 128–141
11. Miller, J. H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. Datsenko, K. A., and Wanner, B. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6640–6645
13. Oh, M. K., Rohlin, L., Kao, K. C., and Liao, J. C. (2002) *J. Biol. Chem.* **277,** 13175–13183
14. Furste, J. P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M., and Lanka, E. (1986) *Gene* (*Amst.*) **48,** 119–131
15. Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P., Givskov, M., and Molin, S. (1998) *Appl. Environ. Microbiol.* **64,** 2240–2246
16. Hyduke, D. R., Rohlin, L., Kao, K. C., and Liao, J. C. (2003) *Omics* **7,** 227–234
17. Tseng, G. C., Oh, M. K., Rohlin, L., Liao, J. C., and Wong, W. H. (2001) *Nucleic Acids Res.* **29,** 2549–2557
18. Almeida, J. S., and Voit, E. O. (2003) *Genome Informatics* **14,** 114–123
19. Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, MA
20. Salgado, H., Santos-Zavaleta, A., Gama-Castro, S., Millan-Zarate, D., Diaz-Peredo, E., Sanchez-Solano, F., Perez-Rueda, E., Bonavides-Martinez, C., and Collado-Vides, J. (2001) *Nucleic Acids Res.* **29,** 72–74
21. Cozzone, A. J. (1998) *Annu. Rev. Microbiol.* **52,** 127–164
22. el-Mansi, E. M., Nimmo, H. G., and Holms, W. H. (1986) *J. Gen. Microbiol.* **132,** 797–806
23. Liao, J. C., Hou, S., and Chao, Y. (1996) *Biotechnol. Bioeng.* **52,** 129–140
24. Peellicer, M. T., Fernandez, C., Badia, J., Aguilar, J., Lin, E. C. C., and Baldoma, L. (1999) *J. Biol. Chem.* **274,** 1745–1752
25. Gimenez, R., Nunez, M. F., Badia, J., Aguilar, J., and Baldoma, L. (2003) *J. Bacteriol.* **185,** 6448–6455
26. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) *Science* **298,** 2390–2392
27. Starai, V. J., and Escalante-Semerena, J. C. (2004) *J. Mol. Biol.* **340,** 1005–1012
28. Nair, S., and Finkel, S. E. (2004) *J. Bacteriol.* **186,** 4192–4198
29. Altuvia, S., Almiron, M., Huisman, G., Kolter, R., and Storz, G. (1994) *Mol. Microbiol.* **13,** 265–272
30. Lazzarini, R. A., Cashel, M., and Gallant, J. (1971) *J. Biol. Chem.* **246,** 4381–4385
31. Barker, M. M., Gaal, T., Josaitis, C. A., and Gourse, R. L. (2001) *J. Mol. Biol.* **305,** 673–688
32. Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B., and Cashel, M. (1993) *J. Bacteriol.* **175,** 7982–7989
33. Teich, A., Meyer, S., Lin, H. Y., Andersson, L., Enfors, S., and Neubauer, P. (1999) *Biotechnol. Prog.* **15,** 123–129
34. Murray, H. D., Schneider, D. A., and Gourse, R. L. (2003) *Mol. Cell* **12,** 125–134
35. Lange, R., and Hengge-Aronis, R. (1994) *Genes Dev.* **8,** 1600–1612
36. Jishage, M., Iwata, A., Ueda, S., and Ishihama, A. (1996) *J. Bacteriol.* **178,** 5447–5451
37. Ball, C. A., Osuna, R., Ferguson, K. C., and Johnson, R. C. (1992) *J. Bacteriol.* **174,** 8043–8056
38. Ali Azam, T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. (1999) *J. Bacteriol.* **181,** 6361–6370
39. Hogema, B. M., Arents, J. C., Bader, R., Eijkemans, K., Yoshida, H., Takahashi, H., Aiba, H., and Postma, P. W. (1998) *Mol. Microbiol.* **30,** 487–498
40. Patnaik, R., Roof, W. D., Young, R. F., and Liao, J. C. (1992) *J. Bacteriol.* **174,** 7527–7532
41. Park, S. J., Cotter, P. A., and Gunsalus, R. P. (1995) *J. Bacteriol.* **177,** 6652–6656
42. Zheng, D., Constantinidou, C., Hobman, J. L., and Minchin, S. D. (2004) *Nucleic Acids Res.* **32,** 5874–5893
43. Kumari, S., Beatty, C. M., Browning, D. F., Busby, S. J., Simel, E. J., Hovel-Miner, G., and Wolfe, A. J. (2000) *J. Bacteriol.* **182,** 4173–4179
44. Beatty, C. M., Browning, D. F., Busby, S. J., and Wolfe, A. J. (2003) *J. Bacteriol.* **185,** 5148–5157

# A Global Regulatory Role of Gluconeogenic Genes in *Escherichia coli* Revealed by Transcriptome Network Analysis

Katy C. Kao, Linh M. Tran and James C. Liao

*J. Biol. Chem. 2005, 280:36079-36087.*
*doi: 10.1074/jbc.M508202200 originally published online August 31, 2005*