Metabolic Characterization of *Escherichia coli* Strains Adapted to Growth on Lactate[∇]

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In comparison with intensive studies of genetic mechanisms related to biological evolutionary systems, much less analysis has been conducted on metabolic network responses to adaptive evolution that are directly associated with evolved metabolic phenotypes. Metabolic mechanisms involved in laboratory evolution of *Escherichia coli* on gluconeogenic carbon sources, such as lactate, were studied based on intracellular flux states determined from ¹³C tracer experiments and ¹³C-constrained flux analysis. At the end point of laboratory evolution, strains exhibited a more than doubling of the average growth rate and a 50% increase in the average biomass yield. Despite different evolutionary trajectories among parallel evolved populations, most improvements were obtained within the first 250 generations of evolution and were generally characterized by a significant increase in pathway capacity. Partitioning between gluconeogenic and pyruvate catabolic flux at the pyruvate node remained almost unchanged, while flux distributions around the key metabolites phosphoenolpyruvate, oxaloacetate, and acetyl-coenzyme A were relatively flexible over the course of evolution on lactate to meet energetic and anabolic demands during rapid growth on this gluconeogenic carbon substrate. There were no clear qualitative correlations between most transcriptional expression and metabolic flux changes, suggesting complex regulatory mechanisms at multiple levels of genetics and molecular biology. Moreover, higher fitness gains for cell growth on both evolutionary and alternative carbon sources were found for strains that adaptively evolved on gluconeogenic carbon sources compared to those that evolved on glucose. These results provide a novel systematic view of the mechanisms underlying microbial adaptation to growth on a gluconeogenic substrate.

Biological systems are capable of adapting to environmental changes by invoking a number of different strategies to achieve optimal overall performance under a specific condition. Laboratory evolution methods have been used to understand adaptive changes in a wide variety of microorganisms (3, 7, 8, 11, 24). Whereas some evolved phenotypes, such as the growth rate or product secretion rate, are readily detectable, the underlying mechanisms that result in improved cellular properties during the evolutionary process are difficult to identify. A number of technologies are now available to help elucidate the underlying mechanistic changes by providing genome-wide measurements of adaptive changes in gene expression or genome sequence (7, 11, 18, 19). While the molecular basis of evolution might be revealed by these technologies, they cannot directly account for the specific metabolic changes responsible for the improved phenotypes. More direct information on metabolic network responses to laboratory evolution is therefore necessary to understand changes in metabolic functions and to link possible genetic mechanisms of evolution to evolved metabolic phenotypes.

The characteristics of metabolic networks can be directly assessed by analyzing metabolic flux distributions in cells ob-

tained over the time course of laboratory evolution. Recently, ¹³C isotopomer-based flux analyses have been successfully applied to investigate metabolic responses and underlying mechanisms in various prokaryotic and eukaryotic systems (4, 9, 12, 26, 29, 33). We have previously studied the metabolic flux states for several gene deletion mutants of *Escherichia coli* that evolved on glucose (16, 20). These flux analyses indicated that these mutant strains adapted to the evolutionary process by activating either latent pathways or pathway of which the major enzyme was deleted. Further studies of gene expression and enzyme activity suggested good correlations between increased pathway fluxes and these measurements. Metabolic flux analysis can therefore provide valuable insight into dynamic responses of the evolutionary system by associating transcriptional or translational changes with evolved metabolic phenotypes.

A large number of laboratory studies of evolution on glucose have been carried out (7, 11, 23, 27, 31), since glucose is the preferred carbon and energy source for most bacteria and eukaryotic cells (2, 28). In comparison to adaptive evolution on glucose, microorganisms might be more capable of adapting to various other carbon compounds in the natural environment. Few studies, however, have investigated the evolutionary changes of metabolic networks with carbon sources other than glucose. In recent years, we have used laboratory evolution to study the adaptation of the *E. coli* K-12 strain and derivative metabolic-gene deletion strains with several alternative carbon sources in addition to glucose. Physiological studies suggest that the effects of most genetic modification, such as growth rates at the end point of evolution, were consistent with that

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predicted by genome-scale metabolic models using flux balance analysis (17). Furthermore, while replicate evolution experiments showed convergence and reproducible growth of *E. coli* on lactate and glycerol, detailed transcriptional studies revealed different gene expression states among evolutionary end points, with only a few genes showing beneficial expression in common across parallel evolution populations (14).

In an effort to further elucidate the metabolic mechanisms involved in the adaptive evolution of bacteria on less-studied gluconeogenic carbon sources, we sought to evaluate metabolic flux states of *E. coli* strains that adaptively evolved in parallel on lactate by using ^{13}C tracer experiments and ^{13}C -constrained flux analysis. Metabolic analyses were conducted at four different time points during laboratory evolution for each of seven populations independently generated using serial passage of exponentially growing cultures in lactate-supplemented minimal medium (14). Adaptive characteristics and mechanisms on lactate were therefore investigated by determining cellular phenotypes, metabolic flux distribution patterns, growth on alternative carbon substrates, and previously generated global gene expression data.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this study were the wild-type *E. coli* K-12 strain MG1655 and seven populations (LacA, LacB, LacC, LacD, and LacE [14] and Lac2 and Lac3 [15]) that adaptively evolved on M9 minimal medium supplemented with L-lactate for up to 60 days in our laboratory. For each evolved population, strains obtained at different days of evolution (day 10 [~100 generations of growth], day 20 [~230 generations], day 40 [~540 generations], and day 60 [~900 generations]) were selected to investigate the effects of adaptive evolution on cellular metabolism. Cultures were grown in 30 ml of M9 minimal medium with 2 g/liter of L-lactate as the carbon source in 250-ml baffled Erlenmeyer flasks using magnetic stir bars for aeration at 30°C. A mixture of 20% (wt/wt) uniformly ^{13}C -labeled L-lactate ($\text{U-}^{13}\text{C}$, >99%; Cambridge Isotope Laboratories, Andover, MA) and 80% (wt/wt) ^{13}C -labeled L-lactate (>99%; Sigma-Aldrich, St. Louis, MO) was used for all ^{13}C labeling experiments. Each culture was inoculated from a preculture with a starting optical density at 600 nm (OD_{600}) of less than 0.005. Cell samples were taken at mid-exponential-growth phase (OD_{600} of ~0.5) for labeling analysis of intracellular fluxes.

Physiological assays. The OD_{600} was measured to monitor batch cell growth. Culture samples were taken periodically, and substrate uptake rates and metabolite secretion rates were measured throughout the course of exponential growth. The depletion of L-lactate and secretion of acetate, ethanol, and other metabolites in the medium were determined using commercial enzymatic assay kits (R-Biopharm AG, Darmstadt, Germany).

Growth on alternative carbon sources was evaluated using the VERSAmax microplate reader system (Molecular Devices Co., Sunnyvale, CA). For each experiment conducted with the VERSAmax system, precultures were grown overnight and allowed to reach mid-exponential-growth phase. The appropriate volume of cultures was used to inoculate the 96-well plates containing 200 μl of medium, yielding an initial OD_{600} of 0.005. Growth on nine different carbon-supplemented M9 minimal media (acetate, alanine, aspartate, glucose, glutamate, glycerol, α -ketoglutarate, pyruvate, and ribose) was tested. The initial carbon content in each medium was approximately 65 to 70 mmol/liter, similar to that used for lactate evolutionary experiments. The plates were incubated at 30°C, and measurements were taken every 15 min with continuous shaking between measurements. Growth rates were averaged across at least three replicate cultures.

GC-MS sample preparation and analysis. Cells growing at mid-exponential-growth phase from 3 to 5 ml of culture were harvested by centrifugation. The cell pellet was washed with 1 ml 0.9% (wt/vol) NaCl and then hydrolyzed in 200 μl of 6 M HCl at 105°C for 24 h. The filtrate of hydrolysate was dried in a heating block at 60°C, and proteinogenic amino acids were derivatized at 85°C for 1 h in 75 μl tetrahydrofuran (Sigma) and 75 μl *N*-methyl-*N*-(tert-butyl)dimethylsilyl trifluoroacetamide (Sigma). After filtration, 3 μl of derivatized sample was injected for the gas chromatography-mass spectrometry (GC-MS) assay. The

GC-MS analysis was performed with a Trace GC/Trace MS Plus system (Thermo Electron Corporation, Waltham, MA), and mass spectral data were obtained for fragments of most derivatized amino acids, as previously reported (20). Prior to analysis of cellular metabolism, the raw mass isotopomer distribution vector (MDV) (fractional abundance set for amino acid fragment with different mass numbers) was corrected for the natural isotope abundances of carbon atoms introduced from the derivatization reagent and all noncarbon atoms involved in the whole fragment (30).

Flux ratios and flux distributions in lactate-grown cells. Intracellular flux states of *E. coli* grown aerobically on lactate were studied based on a metabolic model describing the whole central metabolism. In contrast to glucose-grown *E. coli* cells, in which the glycolytic pathway is predominantly employed for carbon catabolism, trioses and hexoses required for biosynthesis are generated through gluconeogenesis in lactate-grown cells. *pps*-encoded phosphoenolpyruvate (PEP) synthase (PPS) and *fbp*-encoded fructose 1,6-bisphosphatase catalyze key steps towards the gluconeogenic synthesis of six-carbon compounds. The model also included metabolic reactions involved in the tricarboxylic acid (TCA) cycle, the pentose phosphate (PP) pathway, carbon anaplerosis, the Entner-Doudoroff (ED) pathway, and pyruvate metabolism as shown elsewhere (20). The model system consists of 26 reactions and is thus underdetermined when only material balance constraints are conducted for 21 metabolites in the model network. Analysis of ^{13}C isotopomer distribution of intermediates was therefore employed to provide additional useful constraints for fast flux determination through the quantification of the relative contributions of individual pathways to the formation of target metabolites (12, 26, 33).

The flux ratio constraints used to facilitate the metabolic analysis of lactate-evolved strains included ratios relating to various metabolic functions, such as gluconeogenesis (PEP from oxaloacetate [OAA] and pyruvate [Pyr] from malate), anaplerosis (OAA from PEP and OAA generated through the glyoxylate bypass), and the degradation of sugar acids (pyruvate generated through the ED pathway). PEP is generally synthesized either from pyruvate via PPS or from OAA via PEP carboxylase (PCK) in lactate-grown *E. coli*. Under the lactate-labeling condition in this study, activation of both the TCA cycle and the glyoxylate bypass resulted in increased fraction abundance for $\text{OAA}_{2,3}$ fragments (the subscript indicates the carbon position) in comparison with the mass distribution of $\text{Pyr}_{2,3}$ fragment, which was therefore used to determine the contributions of individual reactions to PEP synthesis. The mass distribution of $\text{OAA}_{2,3}$ was derived from the mass distributions for acetyl-coenzyme $\text{A}_{1,2}$ ($\text{CoA}_{1,2}$) ($\text{ACA}_{1,2}$) and α -ketoglutarate $_{2,5}$ ($\text{AKG}_{2,5}$), and the MDV of phenylalanine $_{2,9}$ ($\text{Phe}_{2,9}$) was employed to estimate the mass distributions of $\text{PEP}_{2,3}$ and erythrose 4-phosphate $_{1,4}$ ($\text{E4P}_{1,4}$) based on least-squares fitting analysis (12).

To calculate the fraction of OAA generated via the anaplerotic reaction catalyzed by PEP carboxylase, the equation derived by Fischer and Sauer (12) was used. The possible contribution of the glyoxylate bypass to OAA generation was neglected in this expression estimating the ratio of OAA from PEP and the fractional labeling of CO_2 simultaneously. In addition, we also used the mass distribution of $\text{OAA}_{1,2}$ to quantify both fractions of OAA through anaplerotic PEP carboxylation and OAA derived via the glyoxylate bypass. In this case, the MDV of $\text{OAA}_{1,2}$ generated from the TCA cycle is the average of the MDVs of $\text{OAA}_{2,3}$ and $\text{ACA}_{1,2}$, and the MDV of $\text{OAA}_{1,2}$ derived through the glyoxylate bypass might be obtained by calculating $0.5 \text{OAA}_{3,4} + 0.25 (\text{OAA}_{1,2} + \text{ACA}_{1,2})$. Both methods showed similar flux ratios of OAA from PEP for most evolved populations with only a few exceptions that might be attributed to a slight activity of the glyoxylate bypass.

The fraction of pyruvate obtained from malate via malic enzyme was determined by comparing the mass distributions of the $\text{Pyr}_{1,2}$ fragment with those for malate $_{1,2}$ ($\text{Mal}_{1,2}$) and lactate $_{1,2}$ ($\text{Lac}_{1,2}$). The MDV of $\text{Pyr}_{1,2}$ was calculated from the fraction of PEP from OAA (f_{PCK}), using the equation $\text{PEP}_{1,2} = f_{\text{PCK}} \times \text{OAA}_{1,2} + (1 - f_{\text{PCK}}) \times \text{Pyr}_{1,2}$. The lower bound of the ratio was evaluated by assuming that malate was formed exclusively through the TCA cycle, where the MDV of $\text{Mal}_{1,2}$ was obtained as the average of the MDVs of $\text{Lac}_{2,3}$ and a two-carbon fragment with the same fractional labeling of Lac_3 for each carbon unit. With the assumption that malate was balanced with oxaloacetate, the MDV of $\text{OAA}_{1,2}$ was then used to obtain the upper bound of the ratio (12). In both cases, the TCA cycle yielded a significantly lowered abundance for the unlabeled $\text{Mal}_{1,2}$ fragment than for the $\text{Pyr}_{1,2}$ fragment.

By assuming that 6-phosphogluconate was exclusively generated from the oxidative PP pathway, the fraction of pyruvate formed through the ED pathway was obtained by comparing the MDV of $\text{Pyr}_{1,3}$ with that of $\text{PEP}_{1,3}$. The fractions of pyruvate with the mass of m (m represents the mass number of the unlabeled fragment) and $m + 2$ should be very low if pyruvate is primarily converted from lactate. PEP might contribute more molecules with the mass $m + 2$ to pyruvate (with lowered fractions for $m + 1$ and $m + 3$ fragments) through consecutive

FIG. 1. Physiological data for unevolved and evolved *E. coli* strains on lactate. (a) averaged growth rate (solid squares) and averaged relative change in growth (open squares); (b) averaged biomass yield (solid circles) and averaged relative change in biomass yield (open circles); (c) individual fitness gains for growth rate; (d) individual fitness gains for biomass yield. The standard deviations for the relative changes in plots a and b are not shown, since individual gains are shown in plots c and d. The standard deviations for evolved populations in plots c and d were less than 5% of the mean values and are not shown for clear illustration.

reactions in gluconeogenesis, the oxidative PP pathway, and the ED pathway. Since the nonoxidative PP pathway and the malic enzyme might also result in the cleavage between carbon atoms of pyruvate, the calculated fraction remains an upper bound.

Similar to those shown by Fischer et al. (13) and Blank et al. (1), the above flux ratios were expressed by relevant metabolic reactions, which therefore provided flux calculation with three independent equality constraints and three independent inequality constraints. Quantification of metabolic flux was then performed from a series of randomly generated starting fluxes to get an optimal flux distribution that met the metabolite balance requirements and flux ratio constraints and reproducibly minimized the objective function as described elsewhere (13).

Transcriptional analysis. The detailed description of transcriptional analysis of gene expression in lactate-grown *E. coli* was given previously (14). Briefly, Affymetrix (Santa Clara, CA) *E. coli* antisense genome arrays were used for all transcriptional analyses. Each experimental condition was tested at least in triplicate on lactate using biologically independent cultures and processed following the manufacturer's recommended protocols. Total RNA was isolated from exponentially growing cells with RNeasy Mini Kits (QIAGEN, Valencia, CA), using RNeasy Protect (QIAGEN) to stabilize samples. Total RNA yields were measured using a spectrophotometer (A_{260}), and quality was checked by visualization on agarose gels and by measuring the sample A_{260}/A_{280} ratio. An appropriate amount of isolated total RNA was then used for cDNA synthesis, fragmentation, and terminal labeling, which were all conducted as recommended by Affymetrix. Raw .CEL files were analyzed using robust multiarray average analysis (21) for normalization and calculation of probe intensities. Log-transformed expression values were then assessed for statistically significant differential expression relative to values for wild-type cultures using $\log_2$ ratios and *t* tests.

RESULTS

In an effort to characterize the *E. coli* strains that adaptively evolved on a gluconeogenic carbon source, such as lactate, a variety of detailed experimental analyses were conducted for seven populations that evolved at different generations in M9 medium supplemented with L-lactate. Detailed studies of physiological characteristics, in vivo flux determination, follow-up gene expression analysis, and growth on alternative carbon

substrates were performed to elucidate metabolic and molecular mechanisms that accompany the enhanced phenotypic characteristics during laboratory evolution and evolutionary differences among individual populations.

Physiological characteristics of lactate-evolved *E. coli*. Overall, all 28 (7 populations $\times$ 4 independent time points) lactate-evolved strains exhibited an improved growth rate, lactate uptake rate, and biomass yield compared with the unevolved wild-type strain. Specific growth rate continued to increase over the course of evolution for all seven evolved populations, resulting in a maximal growth rate of $0.51 \pm 0.04 \text{ h}^{-1}$ (more than twofold that of the unevolved strain) at the end point of adaptive evolution (Fig. 1a). An average 50% increase in biomass yield was also observed for end points of evolution (Fig. 1b). Whereas both physiological parameters kept increasing over the 60-day (about 900 generations) evolutionary course, approximately 60% phenotypic improvements were achieved within the first 20 days (less than 250 generations) of evolution, indicating rapid fitness gains in the initial stage of evolution (10). Generally, the relative change in fitness, calculated from the changes in the growth rate or biomass yield between adjacent days studied, decelerated sharply over the course of laboratory evolution. Only minor improvements were observed during the last 20 days (the last 350 generations) of evolution. However, large differences in fitness gains for both physiological measurements were observed, especially in the early stage of evolution, among seven evolved populations (Fig. 1c and 1d).

The major carbon overflow in *E. coli* growing aerobically on lactate was acetate, which was secreted at about 0.4 mmol/mmol lactate by the unevolved strains. The acetate secretion rate, however, declined in most evolved populations, and an up to 50% decrease was observed for some strains. Ethanol, an-

FIG. 2. Flux changes for seven *E. coli* populations that evolved on lactate. Top to bottom in box: LacA, LacB, LacC, LacD, LacE, Lac2, and Lac3. Absolute change in absolute flux for lactate uptake (mmol/g dry cell weight/h) is shown in the *lldD* box, whereas absolute lactate uptake flux in the unevolved strain is shown underneath the *lldD* box in panel a. Absolute change in relative flux that is normalized to the corresponding lactate uptake rate (%) is shown for other selected central metabolic reactions, whereas relative fluxes in the unevolved strain are given underneath individual boxes in panel a. (a) Changes between unevolved strain and day-10 strains (the first 100 generations of evolution); (b) changes between day-10 strains and day-20 strains (the next 130 generations of evolution); (c) changes between day-20 strains and day-60 strains (the last 670 generations of evolution). Corresponding enzymes of representative genes shown above the boxes are as follows: *aceE*, pyruvate dehydrogenase complex; *ackA*, acetate kinase; *adhE*, alcohol dehydrogenase; *eno*, enolase; *glcA*, citrate synthase; *lldD*, L-lactate dehydrogenase; *pck*, phosphoenolpyruvate carboxykinase; *ppc*, phosphoenolpyruvate carboxylase; *pps*, phosphoenolpyruvate synthase; *sucB*, α -ketoglutarate dehydrogenase complex.

other metabolite derived from intracellular acetyl-CoA, was also secreted but at very low concentration levels by wild-type *E. coli*. Interestingly, ethanol secretion gradually increased, but no more than 0.1 mmol/mmol lactate was observed over the course of evolution (data not shown). Moreover, regardless of the evolution, neither TCA-cycle metabolites nor glycerol was detected in lactate-grown *E. coli* strains.

Metabolic flux states over the course of evolution on lactate.

In an effort to understand changes in metabolic mechanisms over the course of laboratory evolution of *E. coli* on lactate, intracellular flux states were quantified from ^{13}C -labeling experiments for all selected evolved populations and unevolved wild-type strains. Flux estimation indicated that the errors for most pathway fluxes were less than 10% for the 95% confidence interval based on the redundant mass distribution data. Fast adaptation of strains on lactate was characterized by up to 80% increases in lactate uptake fluxes within the first 20 days of evolution, whereas only minor changes in lactate utilization were observed during the late stage of adaptive evolution (*lldD* boxes; Fig. 2). Consequently, metabolic capacities of most reactions were enhanced, as shown by hierarchical clustering of fluxes relative to those of the wild-type strain (Fig. 3). The flux of reactions catalyzed by malic enzyme, flux through the glyoxylate bypass, and flux through the ED pathway were excluded from clustering because of extremely low fluxes for the wild-type *E. coli* and insignificant flux changes in evolved populations. Neither the reaction through the malic enzyme nor the reaction through the ED pathway was active in all cases, which is probably attributable to a high intracellular pyruvate pool size in cells growing on lactate. Whereas fitness gains for growth rates and biomass yields varied largely among evolved populations obtained at day 10 (100 generations) of evolution,

relative flux distributions of most day-10 strains fell into the same cluster, suggesting similar overall metabolic responses to selection pressure of a high growth rate at the very early stage of lactate evolution. In contrast, relatively wide spectra of relative flux distributions were observed for strains that evolved for more than 20 days.

During the early phase of evolution, two growth patterns were observed for *E. coli* adaptively growing on lactate. Five of seven parallel evolved populations (LacA, LacB, LacC, LacD, and LacE) exhibited maximal gains for biomass yield between day 10 and day 20 of evolution, which is probably attributable to the decreases in acetate overflow and increases in the TCA cycle flux that therefore provided cells with more precursors and energy for biosynthesis of cellular constituents (Fig. 2b). Among the five strains mentioned above, LacC and LacD had the most significant repression of acetate flow (approximately a 50% drop from day 10 to day 20), resulting in higher fitness gains for biomass yield (30 to 50% increases) and consequently higher evolutionary gains for the growth rate (40 to 80% increases) (Fig. 1c and d). Aside from these strains, maximal gains for biomass yields and growth rates of Lac2 and Lac3 populations were observed even earlier (before day 10 of evolution), with similar patterns of flux rerouting (Fig. 2a). Interestingly, biomass yield of both evolved populations decreased during the next 10 days without significant changes in flux distribution (Fig. 1d and 2b). Despite diverse evolutionary paths in the early phase, the evolutionary end points were convergent for all evolved populations, with much lower gains for both the cell yield and the growth rate during the late evolutionary stage, where a general increase was found for the relative overflow fluxes (Fig. 2c).

FIG. 3. Hierarchical clustering of relative fluxes using Euclidean distance and average linkage methods for evolved *E. coli* strains adaptively grown on lactate. Fluxes were compared with those in the wild-type strain, and the $\log_2$ ratio is used for clustering analysis. Seven parallel evolved populations (LacA, LacB, LacC, LacD, LacE, Lac2, and Lac3) at day 10, day 20, day 40, and day 60 of evolution were analyzed. Clustering analysis was conducted on typical metabolic reactions with significant relative fluxes, as shown in the schematic network map. ACE, acetate secretion; BIOSYN, biosynthesis; CS, citrate synthase; ETH, ethanol secretion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KDH, α -ketoglutarate dehydrogenase complex; LAC, lactate uptake; MDH, malate dehydrogenase; PCK, phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase complex; PPC, phosphoenolpyruvate carboxylase; PPS, phosphoenolpyruvate synthase.

Transcriptional and metabolic responses for genes related to lactate metabolism. To provide more-comprehensive views of evolutionary growth on lactate, both metabolic pathway and transcriptional responses in lactate metabolism- and gluconeogenesis-related genes were dissected based on the flux states and gene expression profiling of the wild-type strain and seven evolved populations obtained at day 20 and day 60 of evolution (14).

(i) Genes for lactate utilization. General increases in transcriptional expression of the *lldD* gene (encoding L-lactate dehydrogenase) were observed for most evolved populations (with the exception of LacC), which was consistent with up to 80% increases in the specific lactate uptake rate for lactate-evolved strains (Fig. 4a). The L-lactate permease-encoding gene *lldP*, which appears in the same operon, *lldPRD*, with the

lldD gene, had similar patterns of transcriptional changes, whereas it is not clear why the expression of another operon gene, *lldR* (encoding operon regulatory protein), did not change significantly. Therefore, lactate uptake flux changes were likely controlled at the transcriptional level over the course of laboratory evolution on lactate.

(ii) Genes associated with pyruvate metabolism. Transcriptional analysis suggested that expressions of pyruvate kinase-encoding genes were largely repressed in lactate-grown wild-type *E. coli*. Transcriptions of these genes were further repressed during evolution on lactate (data not shown). On the contrary, expressions of the gluconeogenic *pps* gene, which encodes PPS, were significantly up-regulated (up to 1.5-fold increase) over the course of evolution (Fig. 4b). The *pps* gene was also the only gene of which the expression was induced in

FIG. 4. Changes in transcriptional expression (upper panel) and absolute metabolic flux (lower panel) of lactate-evolved *E. coli* strains relative to the wild-type strain ($\log_2$ ratio). Expression and flux states are shown for seven populations at day 20 (white bar) or day 60 (gray bar) of evolution. Genes and reactions shown in the figure are (a) *lldD* and lactate dehydrogenation; (b) *pps* and PEP synthesis from pyruvate; (c) *aceE* and pyruvate dehydrogenation; (d) *ppc* and PEP carboxylation; (e) *pck* and oxaloacetate decarboxylation.

all evolved populations, as concluded previously (14). Interestingly, we detected only small increases in absolute flux through PPS for most evolved populations (Fig. 4b). In addition, the flux of pyruvate dehydrogenation (the initial metabolic reaction of oxidative degradation of pyruvate) was generally increased, albeit moderately, in most evolved populations, whereas expression of genes involved in the *pdhR-aceEF-lpdA* operon was not necessarily elevated during evolution (Fig. 4c). The lack of correlation between gene expression and metabolic flux associated with pyruvate metabolism might be related to the energetic demand for cell growth on the nonpreferred carbon substrate of lactate, where pyruvate catabolism through the TCA cycle in evolved strains might be stimulated by low energy charge values (6).

(iii) Anaplerotic and gluconeogenic genes. The PEP-pyruvate-oxaloacetate node acts as an important switch point for carbon flux distribution by controlling the carbon flux into gluconeogenesis, the TCA cycle, and the anaplerotic pathways (25). With the exception of the *pps* gene previously discussed,

transcription of two other groups of gluconeogenic genes, PCK-encoding *pckA* and malic enzyme-encoding *sfcA* and *maeB*, were generally decreased. Metabolic flux through malic enzyme was, irrespective of evolution, extremely low. The flux of the reaction catalyzing the conversion of oxaloacetate to phosphoenolpyruvate was, however, increased markedly in most cases (Fig. 4e). Similarly, despite general down-regulation of *ppc* gene expression, anaplerotic PEP carboxylation flux was accelerated up to twofold for seven parallel evolved strains (Fig. 4d). While the mechanism for the inconsistency between transcriptional and metabolic responses for both genes is unclear, the adjustment of energetic flow through phosphorylation of oxaloacetate by PCK, at least in part, accounted for the flux increases in OAA decarboxylation and PEP carboxylation over the course of lactate evolution. Actually, activities of both enzymes might be largely regulated by several intracellular effectors, as analyzed for glucose-grown conditions (32) and summarized by Sauer and Eikmanns (25). Moreover, while analysis of the *ppc* gene and the *pckA* gene showed similar evolutionary patterns (i.e., decrease in transcription and increase in metabolic flux), the ratio of the PEP carboxylation flux to the OAA decarboxylation flux varied from 1.3 to 3.0 in the evolved populations (2.2 in the wild-type strain). The flexibility of the PEP-oxaloacetate node during evolution might be

important for energetic and anabolic purposes under the selective pressure of rapid growth on lactate.

(iv) Transporter genes of carbon substrates. As previously stated, the transcription of both the lactate/proton symporter-encoding gene *lldP* and the lactate dehydrogenase-encoding gene *lldD* were generally enhanced during evolution on lactate, which was then corresponding well to markedly improved lactate uptake rates of the evolved populations. However, a general decrease in transcription of genes relating to the utilization of many other carbon substrates was observed. Expression levels of the *pstI* and *pstH* genes (encoding the sugar-nonspecific proteins enzyme I and Hpr in the phosphotransferase system) were about one-fourth lower than that for the unevolved strain. A similar down-regulation in expression was observed for most sugar-specific phosphotransferase system enzyme II genes (data not shown). For genes that encode ABC transporters, except for the slight up-regulation of some amino acid ABC transporter genes (e.g., glutamate and glutamine), a general down-regulation of transcription was found for genes of most sugar (e.g., ribose, galactose, and xylose), peptide, and other amino acid (e.g., leucine and isoleucine) transporters. The expression of genes encoding ion channels and most secondary transporters did not change significantly over the course of evolution, whereas down-regulation was observed for the glycerol channel-encoding gene *glpF* and the *dctA* gene, which encodes a dicarboxylate/cation symporter (data not shown). In the wild-type strains growing on lactate instead of glucose, the expression levels of a large number of genes for utilizing unavailable carbon compounds were also up-regulated, as also was observed for cells growing on other poor-quality carbon sources (22). Our results furthermore indicated that during adaptive growth on the nonpreferred carbon source, cells are likely to settle for optimizing their growth on the available substrate by shutting off any potential energy drains, including those involved in the expression of alternative transporters.

Growth of lactate-evolved strains on alternative carbon substrates. To assess the growth characteristics of lactate-evolved *E. coli* strains on nonevolutionary carbon substrates, growth rates were measured for the wild-type and the selected evolved strains on nine alternative carbon substrates, including several amino acids. Overall, on each carbon substrate tested, nearly all evolved populations showed continuous improvements in the growth rate over the course of evolution (Fig. 5). Although some growth gains were lower than those in our previously study (14) because of different microculture systems used, we still observed an overall growth increase of 65% for end points of evolution. Notably, similar to the growth improvement on lactate, specific growth rates of lactate-evolved strains increased more than onefold on pyruvate. Interestingly, similar to the evolutionary growth on lactate, an average more than 70% growth increase was obtained with strains that evolved for less than 20 days (Fig. 5). In particular, despite variable growth adaptation of individual populations with different carbon substrates, the average growth gains were approximately 18% between the wild-type and evolved strains at day 10, 26% between evolved strains at day 10 and day 20, and only 2% to 5% between strains evolved more than 20 days. Moreover, the elevated growth rates, together with probably decreased substrate uptake rates as indicated by the down-regulation of

FIG. 5. Growth characteristics of lactate-evolved strains on alternative carbon substrates. Growth rate measurements for each evolved population were compared with those of the wild-type strain. The relative increases in growth rate are shown for populations at the end point of evolution (gray bar) or populations obtained in the early stage of evolution (day 20; white bar).

relevant transporter genes, might result in significantly improved biomass yields of all evolved populations on alternative carbon substrates.

DISCUSSION

The metabolic network of *E. coli* adaptively evolved on lactate was studied based on ^{13}C tracer experiments and ^{13}C -constrained flux analysis. Although large metabolic flux variations were observed for seven parallel evolved populations at four selected days of evolution, there was no new metabolic pathway response found regarding evolutionary effects on lactate. The main feature of evolution was the overall increased fluxes for already-active central pathways, an effect also revealed from the evolution of several knockout mutants (16, 20). In addition, flux states varied significantly among seven populations that evolved over the observed time frame, especially among strains generated during the early stage of evolution. Examples are the up to 1.6-fold variation in absolute lactate uptake flux and more than 3-fold flux variation in a reaction catalyzed by gluconeogenic PCK.

While the overall metabolic flux states varied markedly among strains that evolved in parallel, the ratio of pyruvate

FIG. 6. Average relative flux for the synthetic flux of key central metabolite of pyruvate, phosphoenolpyruvate, acetyl-CoA, or oxaloacetate (from left to right) for the wild-type or lactate-evolved strains. Enzyme or reaction names are shown in Fig. 3.

dehydrogenation flux to flux of lactate dehydrogenation remained almost unchanged, especially during the early evolutionary phase (Fig. 6). Approximately two-thirds of the carbon source obtained from lactate oxidation was catabolized through the pyruvate dehydrogenase complex for energy requirements. The almost invariable partitioning between gluconeogenic and catabolic fluxes during growth on lactate might be well regulated by the PEP pool size and cellular energy charge (5) and suggests the critical role of the pyruvate node in maintaining a balanced supply of biosynthetic precursors and energy for *E. coli* cells that adaptively evolved on lactate. Early studies on the partitioning of pyruvate in lactate-grown *E. coli* reported a wide range for the ratio of pyruvate dehydrogenase activity to that of PPS (from as high as 4 in the early stage of culture to about 0.5 in late logarithmic phase) (5). Our results indicate that during mid-logarithmic growth, the ratio of pyruvate degradation through the dehydrogenase complex to pyruvate used for gluconeogenesis and anaplerosis was kept at 2.4 ± 0.6 in both wild-type and lactate-evolved *E. coli* cells. The high ratio was likely attributable to a relatively low energy charge in the cells. Actually, based on the fluxes calculated in this study, we observed a more than 1.7-fold energy requirement for all cell processes, including rapid growth on lactate compared to the exponential growth on glucose, indicating possible energy limitation in cells grown on a gluconeogenic carbon source, such as lactate.

In contrast to invariable flux distributions around the pyruvate node, large variations were found for fluxes around the PEP, acetyl-CoA, and oxaloacetate nodes in strains that evolved in parallel. Generally, the ratio of anaplerotic PEP carboxylation flux to PEP synthetic fluxes through gluconeogenic PPS and PCK increased to replenish TCA cycle intermediates that were withdrawn for anabolism. Whereas an average increase of 30% was shown for this ratio, the ratio varied from 0.3 to 0.6 in all evolved populations (Fig. 6). Total overflow (acetate and ethanol) fluxes, however, decreased from about 60% to 45% of pyruvate dehydrogenation flux, which therefore allowed more acetyl-CoA oxidation via the TCA cycle for energy generation. The relative oxaloacetate decarboxylation flux to all oxaloacetate synthesized was also increased by more than 30% on average but with large variation among independently evolved populations (Fig. 6). The ob-

served changes in the metabolic network suggested that the bacterial cells are able to adapt to a new environment by rapidly examining metabolic functions of central metabolic nodes and determining the extent of variations in flux distribution pattern around these nodes. The relative fluxes around metabolites that are critical in both carbon and energy metabolism might not change significantly (e.g., fluxes around pyruvate in lactate metabolism), while the cells could invoke various metabolic strategies to adjust flux distributions on other flexible metabolic nodes to achieve high fitness gains.

Transcriptional and flux studies of lactate-evolved *E. coli* strains revealed that with a few exceptions, qualitative correlations were not found between the overall gene expression and metabolic flux changes, as is partly shown in Fig. 4. There were large variations in transcriptional changes even among strains that had evolved for the same length of time. Moreover, gene expressions of independently evolved populations were not convergent over the course of lactate evolution, as indicated by expression differences among strains evolved at the end point of evolution. Random spontaneous mutations in the *E. coli* genome (18, 19) and complex gene expression responses to highly dynamic evolutionary processes probably account for significant transcriptional variations among *E. coli* strains that evolved in parallel. While flux variations among evolved populations were also found for most pathway reactions, laboratory evolution resulted in overall increases in pathway fluxes that correlated tightly with enhanced lactate utilization and an improved growth rate. While some genetic modifications might provoke modified flux distributions during the course of adaptive evolution, detailed regulation remains unknown. It is clear that multiple layers of information, such as accurate metabolite analysis, are useful in explaining the incoherencies between gene expression and pathway flux and in finding possible changes in regulation phenomena. Such an enhanced global approach would significantly improve the chances of identifying the key causal mechanisms underlying cellular adaptive responses.

Laboratory evolution of *E. coli* in lactate-supplemented medium revealed significant increases in growth on lactate for all populations that evolved in parallel. Similarly, large fitness gains for cell growth were also found for the adaptive evolution of *E. coli* on other gluconeogenic carbon sources. Examples are a 150% increase while evolving on glycerol (14), an above-110% increase on pyruvate, and an approximately 50% increase on α -ketoglutarate (15). The growth of glucose-evolved strains was, in contrast, enhanced by only 20% relative to that of wild-type cells grown on glucose. Significant metabolic adjustment, including enhanced capacity for substrate utilization and optimized intracellular flux distributions, might account for the large increase in evolutionary growth on gluconeogenic carbon sources, as shown with lactate-evolved strains. In contrast, flux analysis of glucose-evolved strains did not show much change in relative flux states during evolution on glucose (unpublished data). In addition, for the evolution on gluconeogenic carbon compounds, most changes in flux distribution and transcriptional expression (14) were shown in the early stage of evolution, resulting in rapid cell adaptation to less-favorable nutrient conditions. The optimal cell metabolic systems obtained through laboratory evolution on lactate were further evidenced by 50 to 100% increases in growth on a wide variety

of nonevolutionary gluconeogenic carbon compounds. Interestingly, the growth of lactate-evolved strains on nongluconeogenic substrates was also largely improved (e.g., $34\% \pm 11\%$ and $63\% \pm 15\%$ increases on glucose and ribose, respectively), suggesting overall improvement of metabolic network characteristics during adaptive growth on lactate. On the contrary, only small growth improvements on alternative carbon sources (e.g., about a 17% increase on lactate and a 47% increase on acetate; unpublished data) were shown for glucose-evolved strains, for which the metabolism did not change much relative to that of the unevolved strain. Therefore, more-comprehensive information and the mechanisms underlying the microbial evolution process can probably be learned by the systematic study of adaptive evolution under conditions with less-optimal metabolic or genetic networks and in appropriate evolutionary stages with most significant fitness gains.

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