Latent Pathway Activation and Increased Pathway Capacity Enable \textit{Escherichia coli} Adaptation to Loss of Key Metabolic Enzymes$^\text{a,5}$

Received for publication, September 12, 2005, and in revised form, November 28, 2005. Published, JBC Papers in Press, November 30, 2005, DOI 10.1074/jbc.M510016200

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The ability of biological systems to adapt to genetic and environmental perturbations is a fundamental but poorly understood process at the molecular level. By quantifying metabolic fluxes and global mRNA abundance, we investigated the genetic and metabolic mechanisms that underlie adaptive evolution of four metabolic gene deletion mutants of \textit{Escherichia coli} ($\Delta pgi$, $\Delta ppc$, $\Delta pta$, and $\Delta tpi$) in parallel evolution experiments of each mutant. The initial response to the gene deletions was flux rerouting through local bypass reactions or normally latent pathways. The principal effect of evolution was improved capacity of already active pathways, whereas new flux distributions were not observed. Combinatorial changes in capacity and pathway activation, however, led to different intracellular flux states that enabled evolution in three of the four parallel cases tested. The molecular bases of the evolved phenotypes were then elucidated by global mRNA transcript analyses. Activation of latent pathways and flux changes in the tricarboxylic acid cycle were found to correlate well with molecular changes at the transcriptional level. Flux alterations in other central metabolic pathways, in contrast, were apparently not connected to changes in the transcriptional network. These results give new insight into the dynamics of the evolutionary process by demonstrating the flexibility of the metabolic network of \textit{E. coli} to compensate for genetic perturbations and the utility of combining multiple high-throughput data sets to differentiate between causal and noncausal mechanisms.

All biological systems are capable of short term response to environmental changes and, on longer time scales, to evolutionary adaptation. Due to the high growth rates and large numbers of individuals, adaptive evolution of microbes under laboratory conditions rapidly leads to improved growth phenotypes. This principle is exploited for evolutionary engineering (1, 2) and experimental testing of general evolutionary principles (3–5). Combining experimental evolution with targeted genetic perturbations then allows one to pose specific questions on robustness and adaptability of biological networks. In response to such externally introduced deletions, microorganisms can invoke a number of different strategies to adjust their functionality. For metabolic networks, these strategies can involve a local bypass of the deleted reaction, complete redirection of flux, reassignment of enzymes to catalyze the deleted reaction, activation of silent genes, or activation of otherwise down-regulated pathways. Whereas all of these mechanisms could potentially cope with genetic perturbations, the exact mechanisms utilized during evolution are poorly understood. Thus, a central question in adaptation and evolution is to determine whether evolving cells refine their existing pathway usage or whether they invoke major metabolic changes such as the activation of latent pathways.

The molecular basis of such evolutionary processes is now experimentally traceable with the ability to rapidly improve microbial phenotypes using laboratory evolution (from weeks to a few months) (6–8) coupled with the principal accessibility of the underlying causes through various “omics” methods or genome resequencing (9). As a particularly popular tool, simultaneous transcript level monitoring of all genes within the genome by DNA microarray technology was used to identify altered gene expression in evolved \textit{Escherichia coli} and \textit{Saccharomyces cerevisiae} strains (4, 10, 11). Altered expression levels, however, do not distinguish between cause and effect and thus cannot directly reveal mechanistic links between altered expression and phenotype. In particular, when considering evolution of metabolic functions, more direct information on intracellular flux rerouting would be necessary to reveal the molecular mechanisms that cause a given improved phenotype. Such \textit{in vivo} reaction rates are accessible through methods of $^{13}$C-based metabolic flux analysis (12), which have been used successfully to identify functional flux states in various microbes (13–17). Potentially, flux data can fill the gap between the intrinsically noisy and indirect transcriptome, proteome, or metabolome data and the actual phenotype (18, 19). Thus, the combination of transcript profiles and quantitative intracellular flux data can provide greater insight into biological processes at the molecular level by implicating gene expression changes to altered phenotypes through association with measured flux data.

Beyond maximizing growth rates of wild-type strains on “exotic” substrates through adaptive evolution (6, 7, 20, 21), rapid recovery of high growth rates was demonstrated for metabolic gene deletion mutants of \textit{E. coli} (3). Replicates of evolved mutants exhibited phenotypic characteristics that suggested the selection of different biochemical mechanisms during parallel evolution under identical conditions. The molecular bases, however, remained unknown because multiple flux scenarios could explain the improved growth phenotypes. Here we evolved four \textit{E. coli} knock-out mutants affected in metabolic key branch points, phosphoglucose isomerase ($pgi$), phosphoenolpyruvate carboxylase ($ppc$), phosphate transacetylase ($pta$), and triose-phosphate isomerase ($tpi$), for several hundred generations under exponential growth conditions on glucose. Since these lesions might be bypassed by at least two different routes, we used metabolic flux and global gene expression data to trace the flux rerouting that enabled evolved strains to maintain high growth rates with highly deleted metabolic pathways.
analysis to identify the metabolic mechanisms responsible for the improved phenotypes.

**EXPERIMENTAL PROCEDURES**

**Strain Construction**—The ppc and tpi mutants of MG1655 were described previously (3), and the pgi and pta mutants were constructed by in-frame gene deletions via homologous recombination facilitated by the λ red recombinase system (22) starting with the E. coli wild-type MG1655 (ATCC, Manassas, VA). The plasmids pKD46, pKD13, and pCP20 were used to introduce the recombinase gene, homologously recombine with the target gene, and remove antibiotic resistance markers, respectively. Each knock-out was confirmed by PCR with genomic DNA.

**Adaptive Evolution**—Evolution of constructed deletion mutants of *E. coli* was conducted in 250 ml of M9 minimal medium supplemented with 2 g/liter of glucose in 500-ml Erlenmeyer flasks using magnetic stir bars for aeration at 37 °C. M9 medium contained (per liter of deionized water) 0.8 g of NH₄Cl, 0.5 g of NaCl, 7.5 g of Na₂HPO₄·2H₂O, and 3.0 g of KH₂PO₄. The following components were sterilized separately and then added (per liter final volume of medium): 2 ml of 1 M MgSO₄, 1 ml of 0.1 M CaCl₂, 0.3 ml of 1 M filter-sterilized thiamine HCl, and 10 ml of a trace element solution containing (per liter) 1 g of FeCl₃·6H₂O, 0.18 g of ZnSO₄·7H₂O, 0.12 g of CuCl₂·2H₂O, 0.12 g of MnSO₄·H₂O, and 0.18 g of CoCl₂·6H₂O. At the start of evolution, initial precultures of each mutant were grown overnight in LB medium before being transferred to minimal medium for adaptive evolution. Duplicate evolution experiments were started from the same parental deletion mutant. In evolution cultures, cells were grown overnight and allowed to reach midexponential growth with an optical density at 600 nm (A₆₀₀) below 0.5 before being diluted by passage into fresh medium. The dilution factor at each passage was adjusted daily to account for changes in growth rate. The optical density was typically at an A₆₀₀ = 2.4 × 10⁻⁶. This process of batch growth and serial passage was conducted for 30 days for the pta mutants (~800 generations), 45 days for the ppc mutants (~750 generations), and 50 days for the pgi (~800 generations) and tpi (~600 generations) mutants, where the ppc and tpi evolution experiments were reported previously (3). This process of evolution resulted in eight evolved mutants with end points designated as *pta*E1, *pta*E2, *ppc*E1, *ppc*E2, *pgi*E1, *pgi*E2, *tpi*E1, and *tpi*E2. The number of generations was estimated on a daily basis by calculating the starting optical density of each batch culture and determining how many doublings occurred during batch growth until being passed into fresh medium. Cultures were evolved until a stable growth rate was achieved for more than 5 days. This process of serial passage maintained a state of prolonged exponential growth so that no culture entered stationary phase. Duplicate cultures were evolved concurrently under identical conditions.

**¹³C-Labeling Experiments**—Frozen glycerol stock cultures were used to inoculate LB complex medium. After 8 h of incubation at 37 °C and constant shaking, LB precultures were used to inoculate M9 medium precultures that were grown overnight for inoculation of cultures for physiological or ¹³C-labeling experiments. Aerobic batch cultures containing 30 ml of M9 medium were inoculated (1:100–1:200) in 500-ml baffled shake flasks and incubated on a gyratory shaker at 250 rpm and 37 °C. For ¹³C-labeling experiments, glucose was added either entirely as the 1-¹³C-labeled isotope isomer (>99%; Euriso-top, GIF-sur-Yvette, France) or as a mixture of 20% (w/w) U-¹³C (>98%; Isotech, Miamisburg, OH) and 80% (w/w) natural glucose.

Cell growth was monitored by following the A₆₀₀. Glucose and acetate concentrations were determined enzymatically using commercial kits (Beckman-Coulter (Zurich, Switzerland) or Dispolab (Dielsdorf, Switzerland)). Other organic acids in culture supernatants were detected by high pressure liquid chromatography analysis (PerkinElmer Life Sciences) at a wavelength of 210 nm, using a Supelcoel C8 column (4.6 × 250 mm) at 30 °C and a mobile phase of 2% (v/v) sulfuric acid at a flow rate of 0.5 ml/min.

The following physiological parameters were determined during the exponential growth phase as described previously (23): maximum growth rate, biomass yield on glucose, specific glucose consumption rate, and specific byproduct production rates, using a predetermined correlation factor of 0.44 g of cellular dry weight per liter and A₆₀₀ unit.

**Metabolic Flux Ratio (METAFoR) Analysis by Gas Chromatography-Mass Spectrometry**—Samples for gas chromatography-mass spectrometry analysis were prepared as described previously (24). Briefly, aliquots of ¹³C-labeled batch cultures were withdrawn during the midexponential growth phase (A₆₀₀ = 0.8–1.2). Cell pellets were hydrolyzed in 6 M HCl at 105 °C for 24 h in sealed microtubes. The hydrolysates were dried under a stream of air at around 60 °C and then derivatized at 85 °C in 30 μl of dimethylformamide (Fluka, Buchs, Switzerland) and 30 μl of N-[(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide with 1% (v/v) tert-butyldimethylchlorosilane (Fluka) for 60 min (25). Derivatized amino acids were analyzed on a series 8000 GC, combined with an MD 800 mass spectrometer (Fisons Instruments, Beverly, MA). The gas chromatography-mass spectrometry-derived mass isotope distributions of proteinogenic amino acids were then corrected for naturally occurring isotopes (24). The corrected mass distributions were related to the in vivo metabolic activities with previously described algebraic equations and statistical data treatment, which quantified 12 largely independent ratios of fluxes through converging reactions and pathways to the synthesis of seven intracellular metabolites (24).

In [1,¹³C]glucose experiments with tpi mutants, ¹³C label occurred in position 3 of pyruvate, but no position 3 ¹³C label occurred in the upstream metabolites phosphoglycerate and phosphoenolpyruvate (PEP). The normal flux ratio definition of METAFoR analysis (24) would relate this label to the Entner-Doudoroff (ED) pathway. This pathway, however, would introduce ¹³C label at the position 1 of pyruvate. The normally inactive methylglyoxal bypass, which channels dihydroxyacetone phosphate (DHAP) molecules to pyruvate, in contrast, would precisely introduce such ¹³C label at the position 3 of pyruvate (26). To quantify the amount of pyruvate originating from DHAP through the methylglyoxal bypass (pyruvate through methylglyoxal bypass), we determined the mass isotopomer distribution vector (MDV) of glycerol, which is identical to the MDV of DHAP₁₋₂ (1–3 indicates that carbon atoms 1–3 of DHAP are considered). The base fragment m₀ = 377, which corresponds to a derivatized glycerol molecule (two tert-butyldimethylsilyl and one dimethylsilyl derivatization chain) was used. To assess the relative contribution of methylglyoxal bypass to pyruvate synthesis (f), the MDV of DHAP₁₋₂, serine₂₋₃, and pyruvate₂₋₃ are used as follows:

\[
\text{Pyruvate}_2-3 = f \times \text{DHAP}_1-2 + (1-f) \times \text{serine}_{2-3} \quad (\text{Eq } 1)
\]

The MDV of DHAP₁₋₂ was not measured; however, the labeling enrichment for this fragment will be similar to the one of DHAP₁₋₂, since position 1 of DHAP will be bearing the ¹³C-labeled atom. Hence, the MDV of DHAP₁₋₂ can be calculated, and Equation 1 can be used to determine f. This approach neglects, however, the contribution of una-

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3 The abbreviations used are: METAFoR, metabolic flux ratio; PEP, phosphoenolpyruvate; ED, Entner-Doudoroff; DHAP, dihydroxyacetone phosphate; MDV, mass distribution vector; EMP, Embden-Meyerhof-Parnas; PP, pentose phosphate.
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beled two carbon molecules coming through the ED pathway. To take the ED pathway contribution into account, we applied an iterative process. The fraction of pyruvate molecules synthesized through the ED pathway was calculated from the previously determined methylglyoxalate flux ratio and the MDV of pyruvate

\[ \text{Pyruvate}_{1–3} = f_1 \text{DHAP}_{1–3} + f_2 \text{MDV (C}_3\text{-molecule with one }^{13}\text{C atom)} + (1 - f_1 - f_2) \text{serine}_{1–3} \] (Eq. 2)

where \( f_1 \) represents the fraction of pyruvate molecules originating from DHAP, \( f_2 \) represents one-half of the fraction derived through the ED pathway, and \((1 - f_1 - f_2)\) represents the fraction derived through serine. Activity of the ED pathway introduces supplementary unlabeled pyruvate

\[ \text{DHAP}_{1–3} = f_1 \text{DHAP}_{1–3} \] (Eq. 3)

where \( f \) represents the contribution of the EMP pathway to DHAP synthesis, and \((1 - f)\) is the contribution of the oxidative branch of the PP pathway to DHAP synthesis. The error on this ratio was determined using error propagation (24).

\[ ^{13}\text{C-Constrained Net Flux Analysis—Intracellular net fluxes were estimated with a stoichiometric model that contained all major pathways of central carbon metabolism (25). For the tpi mutants, the previously described stoichiometric model was augmented with the methylglyoxal bypass based on the MTAForR results. The network considered was similar to the one depicted in Fig. 1. For all mutant analyses, the deleted reactions were kept in the network to obtain independent evidence for their in vivo absence (or evidence for the takeover by another gene). Only for the ppc mutants was the reaction from 2-oxoglutarate to fumarate removed from the network, since METAFoR analysis demonstrated a complete absence of cyclic tricarboxylic acid cycle operation (see Supplemental Table 1). The reaction matrix consisted, for the different strains, of 25–29 unknown fluxes and 21–24 metabolite balances (including the three experimentally determined rates of glucose uptake, acetate, and biomass production).

To solve this underdetermined system of equations with 4–5 degrees of freedom, the following seven calculated flux ratios were used as additional constraints, as was described previously (25): serine derived through the EMP pathway, pyruvate derived through the ED pathway, oxaloacetate originating from PEP, PEP originating from oxaloacetate, pyruvate originating from malate (upper and lower boundaries), and PEP derived through the PP pathway (upper boundary). The first four ratios were used as equality constraints, whereas the others were used only as boundary constraints. When active, based on the METAFoR data, the glyoxylate shunt was also considered in the network, and the ratio oxaloacetate originating from glyoxylate was implemented as an upper bound. The ratios DHAP derived through EMP and pyruvate through the methylglyoxal bypass were used as equality constraints for the tpi mutants, using the following equations: the fraction of pyruvate derived through the methylglyoxal bypass.

\[ a = \frac{V_{\text{DHAP} \rightarrow \text{PYR}}} {V_{\text{DHAP} \rightarrow \text{PYR}} + V_{\text{PEP} \rightarrow \text{PYR}} + V_{\text{MAL} \rightarrow \text{PYR}} + V_{\text{6PG} \rightarrow \text{PYR}}} \] (Eq. 4)

\[ b = \frac{V_{\text{F6P} \rightarrow \text{GAP-DHAP}} - V_{\text{F6P} \rightarrow \text{E4P}} - V_{\text{E4P}} + V_{\text{GAP} \rightarrow \text{F6P}} + V_{\text{GAP} \rightarrow \text{E4P}} + V_{\text{6PG} \rightarrow \text{PYR}}}{V_{\text{F6P} \rightarrow \text{GAP-DHAP}}} \] (Eq. 5)

Fluxes into biomass were calculated from the known metabolite requirements for macromolecular compounds (28) and the growth rate-dependent RNA and protein content (29). The sum of the weighed square residuals of the constraints from both metabolite balances and flux ratios was minimized using the MATLAB function fmincon, with the constraint that the sum of the elements of a mass distribution is equal to 1. These new mass distribution vectors were used to determine the two ratios by repeating the process 1000 times in a MATLAB-based program. The mean values and the S.D. values for the two ratios were determined from these 1000 estimations. The mean values were within 1% of the calculated ratios, and the S.D. value was used as the error for the ratio.

Moreover, in the absence of triose phosphate isomerase in the tpi mutants, the hypothesis used to calculate the flux ratios PEP through the pentose phosphate (PP) pathway and serine through Embden-Meyerhof-Parnas (EMP) pathway is not valid anymore. Therefore, a [1-13C]glucose experiment was used to determine the relative contribution of the EMP pathway to DHAP synthesis. In such a setup, all DHAP molecules derived through the EMP pathway contain a 13C atom at position 1, whereas the PP pathway will generate unlabeled DHAP molecules. Therefore, the relative contribution of the EMP pathway to DHAP can be determined as follows.

\[ \text{DHAP}_{1–3} = f(MDV of C}_3\text{ with one }^{13}\text{C atom)} + (1 - f) \text{MDV of labeled C}_3 \] (Eq. 3)

Fluxes into biomass were calculated from the known metabolite requirements for macromolecular compounds (28) and the growth rate-dependent RNA and protein content (29). The sum of the weighed square residuals of the constraints from both metabolite balances and flux ratios was minimized using the MATLAB function fmincon. The residuals were weighed by dividing through the experimental error (25). The computation was repeated at least five times with randomly chosen initial flux distributions to ensure identification of the global minimum, and the system always converged to the same solution. An extended version of the software FiaFItFlux was used to calculate all metabolic flux ratios and net fluxes (26).

mRNA Transcriptional Profiling—Affymetrix (Santa Clara, CA) E. coli antisense genome arrays were used for all transcriptional analyses. Each experimental condition was tested in triplicate using independent cultures and processed following the manufacturer’s recommended protocols. Six replicates of the wild-type strain grown on glucose were used for the reference point. Briefly, cultures were grown to midexponential growth phase \((A_{660} \approx 0.5)\). 3 ml of culture was added to 6 ml of RNAprotekt (Qiagen, Valencia, CA), and RNA was isolated using RNeasy kits (Qiagen, Valencia, CA) following the manufacturer’s instructions. Total RNA yields were measured using a spectrophotometer \((A_{260})\), and quality was checked by visualization on agarose gels and by measuring the sample \(A_{260}/A_{230}\) ratio. cDNA synthesis, fragmentation, and terminal labeling were conducted as recommended by

\[ ^4 A. Perrenoud, A. Schicker, and U. Sauer, submitted for publication. \]
Expression values were then assessed for statistically significant differential expression using t tests. After conducting pairwise t test comparisons between evolved mutants and wild type, those genes meeting a 5% false discovery rate-adjusted p value cut-off were chosen as having statistically significant changes in gene expression. This resulted in selection of subsets of differentially expressed genes for each tested evolution mutant.

These subsets of differentially expressed genes were then organized in known regulon structures (32) for further analysis. The probability (p value) of the observed regulon enrichment of differentially expressed genes was calculated using the hypergeometric distribution (33),

$$ p = 1 - \sum_{i=0}^{y-1} \frac{\binom{r}{i} \binom{N-r}{n-i}}{\binom{N}{n}} $$

(Eq. 6)

where N (equal to 4345) represents the total number of E. coli genes listed on the Affymetrix GeneChip, r is the total number of genes that are a part of the regulon, n is the number of differentially expressed genes, and y is the number of genes that are differentially expressed and a member of the regulon.

**In Vitro Enzyme Activity**—Crude cell extracts for in vitro enzyme assays were prepared from pellets of 45-ml culture aliquots. Pellets were resuspended in 4 ml of 0.9% (w/v) NaCl and 10 mM MgSO4, passed three times through a French pressure cell (1.2 × 107 pascals), and centrifuged at 11,000 × g. In vitro activity of the methylglyoxal bypass was determined by enzyme assays for methylglyoxal reductase and two coupled enzyme assays for methylglyoxal synthase-methylglyoxal reductase and methyglyoxal synthase-glyoxalase. For methylglyoxal reductase, the assay of Saikusa et al. (34) was adapted: 50 mM Tris-HCl (pH 7.5), 0.125 mM NADPH, 10 mM methyglyoxal, and 100 μl of crude cell extract per ml of assay. The assay for methylglyoxal synthase-methylglyoxal reductase was similar to the previous one, with the sole difference being the use of 0.75 mM DHAP instead of methyglyoxal. For methylglyoxal synthase-glyoxalase, the assay of Hoper and Cooper (35) was adapted: 40 mM imidazole (pH 7.0), 1.65 mM glutathione (pH 7.0), 0.75 mM DHAP, and 100 μl of crude cell extract per ml of assay. For all three assays, the reaction was initiated by the addition of either methyglyoxal or DHAP and consumption of NADPH (at 340 nm), and formation of S-lactoylglutathione (240 nm) was detected in the two first and second assays, respectively. The extinction coefficients for NADPH and S-lactoylglyoxalase were 6.2 and 3.4 mM−1 cm−1, respectively. The protein content in crude cell extracts was determined with the biuret reaction.

**RESULTS**

**Physiological Characterization of Evolved Knock-out Mutants**—To identify the metabolic routes chosen by adaptive evolution to cope with a gene knock-out, we selected four gene knock-outs that severely affect the flux distribution at key branch points of glucose catabolism: pgI, ppc, pta, and tpi (Fig. 1). Two parallel cultures of each mutant were evolved for 30–50 days (600–800 generations), under conditions of exponential batch growth in M9 minimal medium with 2 g/liter glucose. The end points of evolution were defined as having unaltered physiology for >100 generations, and these mutant populations were designated as ptaE1, ptaE2, ppcE1, ppcE2, pgIE1, pgIE2, tpiE1, and tpiE2. Akin to the previously reported ppc and tpi evolution experiments (3), the pgI and pta mutants rapidly evolved improved phenotypes (Table 1). Most knock-out mutations severely reduced the specific growth and glucose consumption rates, but all evolved mutants largely recovered the wild type-like rates at the end point of evolution. Whereas all mutants evolved to improved phenotypes, some parallel evolved cultures displayed different phenotypes; e.g. the evolved pgI mutants pgIE1 and pgIE2 exhibited very different overflow metabolism and biomass yield, and the ptaE1 and ptaE2

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**TABLE 1**

Aerobic growth parameters of parent mutant, evolved mutants, and wild type during exponential growth on glucose

| Strain | Day of evolution | Growth rate | Biomass yield | Glucose consumption rate | Acetate production rate | Pyruvate production rate |
|--------|-----------------|-------------|---------------|-------------------------|------------------------|-------------------------|
|        |                 | h−1         | g CFU ml−1 h−1| mmol g−1 h−1           | mmol g−1 h−1           | mmol g−1 h−1           |
| Wild type | 0              | 0.63 ± 0.03 | 0.40 ± 0.02  | 8.8 ± 0.5               | 4.5 ± 0.7              | 0.0 ± 0.0               |
| pgI    | 0              | 0.17 ± 0.00 | 0.41 ± 0.02  | 2.3 ± 0.1               | 0.1 ± 0.1              | 0.0 ± 0.0               |
| pgIE1  | 50             | 0.34 ± 0.06 | 0.32 ± 0.07  | 5.8 ± 0.3               | 2.6 ± 0.5              | 0.0 ± 0.0               |
| pgIE2  | 50             | 0.53 ± 0.03 | 0.53 ± 0.02  | 5.6 ± 0.5               | 0.0 ± 0.0              | 0.0 ± 0.0               |
| ppc    | 0              | 0.22 ± 0.01 | 0.40 ± 0.01  | 3.0 ± 0.0               | 1.1 ± 0.0              | 0.0 ± 0.0               |
| ppcE1  | 45             | 0.55 ± 0.04 | 0.37 ± 0.02  | 8.1 ± 0.1               | 2.2 ± 0.3              | 0.0 ± 0.0               |
| ppcE2  | 45             | 0.56 ± 0.01 | 0.39 ± 0.01  | 7.8 ± 0.3               | 2.2 ± 0.2              | 0.0 ± 0.0               |
| pta    | 0              | 0.58 ± 0.02 | 0.36 ± 0.02  | 9.1 ± 0.9               | 0.6 ± 0.2              | 4.3 ± 2.1               |
| ptaE1  | 30             | 0.64 ± 0.04 | 0.34 ± 0.01  | 10.3 ± 0.6              | 0.7 ± 0.3              | 4.6 ± 2.2               |
| ptaE2  | 30             | 0.66 ± 0.00 | 0.43 ± 0.02  | 8.6 ± 0.5               | 0.7 ± 0.5              | 2.8 ± 2.5               |
| tpi    | 0              | 0.18 ± 0.02 | 0.33 ± 0.02  | 2.7 ± 0.0               | 0.2 ± 0.1              | 0.0 ± 0.0               |
| tpiE1  | 50             | 0.51 ± 0.02 | 0.36 ± 0.01  | 7.8 ± 0.8               | 1.0 ± 1.0              | 0.0 ± 0.0               |
| tpiE2  | 50             | 0.49 ± 0.02 | 0.37 ± 0.02  | 7.3 ± 0.3               | 0.9 ± 0.9              | 0.0 ± 0.0               |

* Average ± S.D. for 3–7 independent experiments.
mutants differed significantly in their glucose uptake rates (Table 1). Thus, the evolutionary end points are convergent and reproducible with respect to the main selective pressure for high growth rate, but the underlying physiological states are not necessarily the same.

Metabolic Flux Analysis—Which are the actual metabolic mechanisms chosen by adaptive evolution, and do all mutants evolved in parallel rely on the same mechanisms to cope with a given lesion? To answer these questions, intracellular fluxes were quantified from $^{13}$C-labeling experiments (25).

As expected (24, 36, 37), glucose catabolism in the unevolved $pgi$ mutant is rerouted from the EMP to the PP and ED pathways to bypass the lesion (Fig. 2A). Furthermore, operation of the otherwise inactive glyoxylate shunt in the unevolved $pgi$ mutant was consistent with earlier reports (37, 38). Whereas the severely altered flux distribution was maintained, evolution more than doubled the absolute flux level to about 65% of the wild-type glucose uptake rate (Table 1). In contrast to the unevolved $pgi$ mutant, initial glucose catabolism proceeded almost exclusively through the PP pathway in both evolved mutants (Fig. 2A).
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In the lower part of metabolism, however, the two evolved network topologies differed significantly. Whereas the pgiE2 mutant flux distribution was similar to the unevolved mutant with an active glyoxylate shunt and no acetate secretion, the pgiE1 mutant was more similar to the wild type with acetate secretion and full tricarboxylic acid cycle flux instead of the glyoxylate shunt flux. Thus, the pgi mutants evolved to improved phenotypes through rather different intracellular flux scenarios. Unexpectedly, these flux adaptations occurred far away from the deleted gene, possibly suggesting the need for downstream metabolic adjustment in relation to the initially implemented flux distribution. Knock-out of phosphoglucone isomerase forced glucose catabolism primarily through the NADPH-producing PP pathway, resulting in NADPH production encompassing the biosynthetic needs of the cells (36). Hence, in contrast to the wild type, where the membrane-bound transhydrogenase PntAB converts NADH to NADPH to meet the NADPH requirements of the cells, in the pgi mutants, the soluble transhydrogenase converts the excess NADPH to NADH (Fig. 2A).

Generally, PEP carboxylase inactivation precludes growth on glucose as the sole carbon source, because this anaplerotic reaction replenishes tricarboxylic acid cycle intermediates that are withdrawn for biosynthesis (39). Physiological suppressor mutants occur rapidly, however, and the slow glucose growth phenotype of the unevolved, suppressed ppc mutant was almost fully recovered through adaptive evolution with indistinguishable physiology in the two evolved mutants (Table 1). In all ppc mutants, the normally glucose-repressed glyoxylate shunt replaced the anaplerotic function of PEP carboxylase (Fig. 2B), as was described previously (40). The flux through the shunt, however, was in 40% excess of the anabolic demand for the biomass precursors oxaloacetate and 2-oxoglutarate. In the unevolved and the ppcE1 mutants, this excess flux was catabolized through the PEP-glyoxylate cycle with the glyoxylate shunt and PEP carboxykinase as key reactions (38). Additionally, malic enzyme contributed to this cycle in all three ppc mutants by converting malate to pyruvate. Thus, the latent glyoxylate shunt functionally replaced the ppc mutation and was immediately invoked upon deletion of ppc. Evolution of the ppc mutants led to subtle differences in the metabolic network topology by using either PEP carboxykinase or malic enzyme as was necessary to balance the excess precursors being generated through the active glyoxylate shunt.

Blocking the main acetate secretion route in pta mutants was counteracted by secreting pyruvate instead of acetate (Table 1). As had been observed for different pta mutants, small amounts of acetate were still produced (41). Since the phenotype of the unevolved mutant was otherwise similar to the wild type, growth physiology and network topology were largely unaltered in the evolved mutants (Fig. 2C and Table 1). Nevertheless, the end point flux states were detectably different in the two evolved pta mutants with significantly lower absolute fluxes and lower pyruvate secretion in the ptaE2 mutant. The increased tricarboxylic acid cycle and EMP pathway expressions, which had been observed for a double pta-ackA knock-out mutant (42), were not reflected in the flux states of the pta mutants.

Knock-out of the triose-phosphate isomerase in the tpi mutant affects a stoichiometrically equal splitting of the glycolytic flux into glyceraldehyde phosphate and DHAP. To prevent internal accumulation of DHAP, tpi mutants convert DHAP to pyruvate through the normally inactive methylglyoxal bypass (26, 43) (Fig. 2D). Since the present 13C data provide only indirect evidence for methylglyoxal bypass fluxes, its activation was confirmed through in vitro enzyme data. Compared with the wild type, the unevolved tpi mutant exhibits about 2.5-fold higher in vitro activities in the glyoxalase I branch of the methylglyoxal bypass (Table 2). Evolution more than doubled the overall flux level to about 80% of the wild-type glucose uptake rate (Table 1). This is probably a direct consequence of improved methylglyoxal bypass fluxes through the glyoxalase I branch with about 4-fold increased in vitro activities (Table 2), indicating that the glutathione intermediate branch is more suitable for higher fluxes through the methylglyoxal bypass than the two consecutive oxidation-reduction reactions in the methylglyoxal reductase branch. Thus, the normally latent methylglyoxal bypass functionally replaced the introduced tpi mutation. Whereas the unevolved strain exhibited slow growth, evolution led to greatly improved growth through implementation of metabolic adjustments (methylglyoxal bypass) downstream of the introduced lesion needed to accommodate different metabolite pools present due to the absence of triose-phosphate isomerase, which resulted in almost tripled absolute fluxes.

| Specific activities | Methylglyoxal reductase | Methylglyoxal synthase and methylglyoxal reductase | Methylglyoxal synthase and glyoxalase I |
|---------------------|-------------------------|--------------------------------------------------|-----------------------------|
| **tpi mutant**      | 53.5 ± 0.3a             | 2.8 ± 1.6                                       | 42 ± 8                      |
| **tpiE1 mutant**    | 24.1 ± 2.5              | 0.5 ± 0.2                                       | 137 ± 10                    |
| **tpiE2 mutant**    | 33.2 ± 2.3              | 0.7 ± 0.7                                       | 194 ± 8                     |
| **MG1655**          | 22.5 ± 0.1              | 1.0 ± 0.3                                       | 16 ± 4                      |

Average and deviation from triplicate experiments.
and, less pronounced, of all glycolytic enzymes in \textit{pgi} \textit{E2} was consistent with the flux data. Moreover, increased expression of the glyoxylate shunt gene \textit{glcB} correlated with the activated shunt in this \textit{pgi} \textit{E2} mutant, and no significant difference in expression was seen in the \textit{pgi} \textit{E1} mutant with an inactive shunt. Thus, there is a genetic basis for the much lower tricarboxylic acid cycle and much higher glyoxylate shunt in the \textit{pgi} \textit{E2} mutant and the absence of significant changes in the \textit{pgi} \textit{E1} mutant.

\textit{E. coli} has two transhydrogenases, a soluble and a membrane-bound, which are used to balance the NADPH and NADH pools (44–46). The soluble transhydrogenase, encoded by \textit{udhA}, converts NADPH to NADH, whereas the membrane-bound transhydrogenase, encoded by \textit{pntAB}, converts NADH to NADPH and accounts, in batch cultures of the wild type, for around 40% of the required NADPH production (36). Expression changes for the two transhydrogenases mostly correlated with the flux distribution for both evolved mutants. Indeed, the expression of the membrane-bound transhydrogenase showed a statistically significant decrease in expression, since NADPH was produced in excess to the biosynthetic requirements, whereas the expression of the soluble transhydrogenase increased, but surprisingly was significant only for the \textit{pgi} \textit{E2} that had the lower NADPH to NADH conversion (Fig. 2A).

In both evolved \textit{ppc} mutants, altered expression of 14 genes was qualitatively correlated with flux changes (Fig. 3B and Supplemental Table 2). Common to both mutants was altered expression in the lower part of central metabolism with decreased expression of \textit{ackA} and the tricarboxylic acid cycle genes \textit{sucABC} and \textit{sdhABCD} and increased expression of \textit{fumB} and the glyoxylate shunt gene \textit{aceA}. Thus, decreased acetate secretion, activation of the glyoxylate shunt, and decreased tricarboxylic acid cycle fluxes were probably directly determined through altered expression of key genes in these pathways.

In the \textit{tpi} \textit{E1} and \textit{tpi} \textit{E2} mutants, 15 fluxes representing 42 genes and 16 fluxes representing 43 genes, respectively, changed significantly when compared with the wild-type. Qualitative correlation between expression and flux changes, however, was observed for only two genes (one of which was decreased expression of \textit{tpi}) in the \textit{tpi} \textit{E1} and four genes in the \textit{tpi} \textit{E2} (Fig. 3C and Supplemental Table 2). Notably, the major flux change in the mutants compared with the wild-type, activation of the normally latent methylglyoxal bypass, was probably genetically determined, because both mutants increased expression of \textit{gloA} (>2-fold). This view is further supported by the about 10-fold higher in vitro activity of the \textit{gloA}-encoded glyoxylase I (Table 2). The decreased glycolytic and increased tricarboxylic acid fluxes were not reflected by changes in gene expression.

Since higher glyoxylate shunt fluxes appeared to have a genetic basis, we were interested to see whether this was due to a particular mechanistic change affecting only the shunt genes or if other expression changes were correlated in individual genes or in a regulatory cascade. Hence, we searched for genes with statistically significant expression changes in the same direction in the \textit{pgi} \textit{E2}, \textit{ppc} \textit{E1}, and \textit{ppc} \textit{E2} mutants with an active glyoxylate shunt. Expression of 38 genes was increased, and expression of 132 genes was decreased exclusively in the \textit{pgi} \textit{E2}, \textit{ppc} \textit{E1}, and \textit{ppc} \textit{E2} mutants but not in the other three mutants investigated. Mostly, these genes were involved in metabolic pathways that branch off from the tricarboxylic acid cycle, including increased expres-
Activation of the glyoxylate shunt mutants affected redox metabolism, with increased expression of the hyaA, torA, torC, torY, and torZ genes involved in quinone biosynthesis from the tricarboxylic acid cycle intermediate α-ketoglutarate. This coordinated pattern of expression changes may indicate one or more mutations in the transcriptional network that controls expression of the glyoxylate shunt and many other genes. Whereas the active glyoxylate shunt was clearly relevant for the observed phenotypes, it remains unclear whether the related changes contribute to the mutant phenotypes.

Changes in transcriptional regulatory units were further investigated by using significant expression changes in each strain to calculate the probability that a statistical change has occurred in a regulon. p-values for each evolved mutant were calculated for 124 regulons using the hypergeometric distribution at a p-value cut-off of 5%. Screening for regulatory changes that could correspond to physiological changes, we found that both evolved pgi mutants exhibited consistent down-regulation of the LeuO regulon (associated with leucine biosynthesis) and that all three mutants utilizing the glyoxylate shunt (pgiE2, ppcE1, and ppcE2) down-regulated the MetJ regulon (associated with methionine biosynthesis) (Table 3). Whereas production of the amino acids leucine and methionine is essential, it appears that a change in the regulatory network was induced to balance the amino acid with other biological demands. In the case of the pgi mutants, lower availability of pyruvate (a precursor to leucine) could force a reduction in leucine production to allow pyruvate to fulfill other metabolic needs. In the case of mutants utilizing the glyoxylate shunt, activation of the PEP(pyruvate)-glyoxylate cycle (38) was found, which involves oxaloacetate (a precursor to methionine) and thus could limit the production of methionine. Thus, in two cases, adaptive mechanisms occurred in the transcriptional regulatory network that are closely connected to the observed changes in the metabolic network.

### DISCUSSION

Several strategies can be invoked in response to externally introduced genetic perturbations that confer genetic robustness to the network. The initial response to the four investigated deletions was a local rerouting of fluxes around the lesion, which involved activation of latent pathways in several mutants. Although cultures were followed for several hundred generations, there was not a single case where evolution invented a new solution (e.g. activation of a silent gene or reassignment of enzyme function). Instead, the primary effect of evolution over the observed time frame was an increase in the capacity of already active pathways. As a consequence, the overall fluxes increased in those mutants that were severely affected by the mutation (pgi, ppc, and tpi mutants). In several cases, the first metabolic response in the unevolved mutants, which involved the activation of pathways that are usually inactive or almost inactive, such as the glyoxylate shunt or the ED pathway, was even lost during adaptive evolution. Examples are the loss of glyoxylate shunt activity in pgiE1 and the two evolved tpi mutants and the loss of local flux rerouting through the ED pathway in the evolved pgi mutants. Hence, the immediate strategy invoked by E. coli in response to an externally introduced genetic perturbation is not always optimal.

| MetJ Regulon | p-value | pg/E1 | pg/E2 | ppc/E1 | ppc/E2 | tpi/E1 | tpi/E2 |
|--------------|---------|-------|-------|--------|--------|--------|--------|
| Gene         | WT Mean | Log2 Ratio |
| b0197        | 11.40   | -0.90 | -2.64 | -0.69  | -0.69  | -0.72  | -1.02  |
| b0198        | 10.19   | -1.31 | -2.02 | -1.78  | -2.07  | -0.88  | -1.59  |
| b0199        | 10.33   | -1.07 | -2.38 | -2.04  | -2.15  | -0.94  | -1.55  |
| b0605        | 11.81   | -0.67 | -2.00 | -1.46  | -2.02  | -0.84  | -1.33  |
| b0606        | 10.02   | -0.43 | -1.07 | -1.21  | -1.17  | 0.16   | -0.21  |
| b2942        | 10.82   | -1.14 | -3.06 | -2.67  | -2.08  | -0.58  | -1.29  |
| b3008        | 8.68    | -0.81 | -1.97 | -1.13  | -0.89  | -0.53  | -0.98  |
| b3939        | 9.08    | -0.45 | -1.65 | -1.56  | -1.99  | -0.17  | -0.91  |
| b3940        | 8.29    | -0.08 | -0.34 | 0.09   | -0.10  | 0.09   | -0.02  |
| b3941        | 10.42   | -1.22 | -2.59 | -2.53  | -2.18  | -0.74  | -1.73  |
| b4013        | 9.76    | -1.01 | -2.84 | -2.52  | -1.88  | -0.66  | -1.13  |

| LeuO Regulon | p-value | pg/E1 | pg/E2 | ppc/E1 | ppc/E2 | tpi/E1 | tpi/E2 |
|--------------|---------|-------|-------|--------|--------|--------|--------|
| Gene         | WT Mean | Log2 Ratio |
| b0071        | 10.73   | -0.87 | -1.46 | 1.58   | 1.32   | -0.19  | 0.04   |
| b0072        | 11.27   | -1.04 | -1.59 | 1.06   | 0.96   | -0.20  | -0.04  |
| b0073        | 11.03   | -1.15 | -1.75 | 0.51   | 0.63   | -0.20  | -0.38  |
| b0074        | 11.38   | -1.26 | -1.71 | -0.40  | -0.06  | 0.04   | -0.32  |
| b0075        | 8.84    | -0.94 | -1.51 | -1.08  | -2.42  | -0.23  | -0.95  |
Adaptive Evolution of E. coli

However, no single optimum exists, as demonstrated by the parallel evolved pgI mutants that differ in anaplerotic reactions and tricarboxylic acid cycle fluxes.

Whereas there are a number of ways for an organism to compensate for a gene deletion, one hypothesis is that an introduced genetic perturbation should force a redistribution of fluxes through the network as an immediate rescue solution, and evolution would then entail a process of refining this newly established initial state (47). Generally, our results fully support this hypothesis, but we were surprised to find relatively large variations in the flux states of parallel evolved mutants. This appears to reflect the robustness of the metabolic network and its ability to utilize different means to not only survive but to improve growth characteristics. These results provide experimental support to computational results stating that bacterial metabolic networks have many different means of achieving similar, equivalent functionality (alternate optimal solutions) (48). It is likely that biological noise and stochastic variations play some role in the development of these diverse flux states, but it is presently unclear how these properties are integrated into evolutionary dynamics, and selection is not clearly defined.

The distribution of molecular fluxes is regulated by multiple mechanisms at several levels that include gene expression, posttranscriptional control, enzyme kinetics, and allosteric control. To determine whether transcriptional modifications were the cause of the altered flux distributions, gene expression levels were systematically compared with flux levels. A particularly high correlation was found for the complete activation or inactivation of latent pathways, such as the glyoxylate shunt and the methylglyoxal bypass. In those cases, expression of at least one gene correlated qualitatively with the flux changes through these pathways. Moreover, flux changes in the tricarboxylic acid cycle also correlated mostly with altered gene expression, e.g. the reduced fluxes in parts of the tricarboxylic acid cycle for pgI and very evolved pgI mutants. The lack of correlation in the tpi mutants might be related to the less pronounced changes in absolute tricarboxylic acid cycle fluxes than for the other three mutants, whose succinyl-CoA synthetase and succinate dehydrogenase fluxes were essentially zero (Fig. 2 and Supplemental Table 3). No flux-expression correlation was found for the third pathways of initial glucose catabolism, not even in the pgI mutants with greatly increased PP pathway fluxes. Solely pgIa and gapA expression correlated with the reduced EMP pathway flux in both pgI evolved mutants. Hence, glyoxylate shunt, methylglyoxal bypass, and tricarboxylic acid cycle flux changes appear to be controlled, at least in part, at the transcriptional level. Flux through the PP and EMP pathways and PEP carboxylase, in contrast, were not controlled at the transcriptional level.

Similarly, a strong qualitative correspondence between gene expression and metabolic fluxes for the glyoxylate shunt had previously been observed in S. cerevisiae, whereas tricarboxylic acid cycle and PP pathway fluxes only partially correlated (49). Comparison of flux and gene expression for E. coli grown under anaerobic conditions on xylose and glucose showed a similar absence of correlation for the PP pathway; however, in contrast to our results, the EMP pathway showed a strong flux-gene expression correlation (27). The absence of correlation in our data might be related to the relatively small changes in absolute EMP pathway fluxes. Hence, gene expression changes are not always manifested in the expressed phenotype, since attributes such as translational efficiency, allosteric control, or changes in enzyme kinetics will not be reflected in individual mRNA transcript levels. Thus, the combined analysis of gene expression data with flux data is one means of pinpointing mRNA transcript and regulatory changes that may be causal to observed phenotypes. In the case of this study, interpreting the gene expression data in the context of flux measurements allowed us to focus on a small number of meaningful expression changes out of the thousands of observed expression changes.

Generally, E. coli is able to rapidly recover to nearly wild-type growth from a severely crippled phenotype resulting from initial genetic perturbations by utilization of existing (though sometimes dormant) pathways parallel to the lesion. This conclusion can probably be extrapolated to mutations in the central metabolism of many organisms that, like E. coli, feature a highly interconnected network of core reactions but may differ for organisms with simpler metabolism and for mutations in less redundant parts of the network (e.g. biosynthetic routes to essential compounds). Metabolic capacity of the activated parallel pathways appears to be analogous to the pathways used in the wild type; however, downstream metabolic adjustments are often needed to refine usage of the new pathways. These adjustments could be implemented to alleviate intracellular metabolite pools resulting from new pathway usage (pgI, pgP, and tpi mutants). It was even observed that strains evolved in parallel frequently utilized different means of achieving this refinement, sometimes leading to surprisingly large differences in flux states (pgI E1 and pgI E2). Overall, we have found that the metabolic network of E. coli is robust in response to a genetic perturbation, with metabolic adjustments occurring in two phases: an initial flux rerouting to compensate for the lesion and a subsequent downstream adjustment to optimize flux rerouting. Combined analysis of multiple data types was pivotal to unravel mechanistic details of these downstream adjustments.

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Latent Pathway Activation and Increased Pathway Capacity Enable *Escherichia coli* Adaptation to Loss of Key Metabolic Enzymes

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*J. Biol. Chem.*, 2006, 281:8024-8033. doi: 10.1074/jbc.M510016200 originally published online November 30, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M510016200

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