Global Transcription and Metabolic Flux Analysis of *Escherichia coli* in Glucose-Limited Fed-Batch Cultivations^{∇†}

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A time series of whole-genome transcription profiling of *Escherichia coli* K-12 W3110 was performed during a carbon-limited fed-batch process. The application of a constant feed rate led to the identification of a dynamic sequence of diverse carbon limitation responses (e.g., the hunger response) and at the same time provided a global view of how cellular and extracellular resources are used: the synthesis of high-affinity transporters guarantees maximal glucose influx, thereby preserving the phosphoenolpyruvate pool, and energy-dependent chemotaxis is reduced in order to provide a more economic “work mode.” σ^S -mediated stress and starvation responses were both found to be of only minor relevance. Thus, the experimental setup provided access to the hunger response and enabled the differentiation of the hunger response from the general starvation response. Our previous topological model of the global regulation of the *E. coli* central carbon metabolism through the *crp*, *cra*, and *relA/spoT* modulons is supported by correlating transcript levels and metabolic fluxes and can now be extended. The substrate is extensively oxidized in the tricarboxylic acid (TCA) cycle to enhance energy generation. However, the general rate of oxidative decarboxylation within the pentose phosphate pathway and the TCA cycle is restricted to a minimum. Fine regulation of the carbon flux through these pathways supplies sufficient precursors for biosyntheses. The pools of at least three precursors are probably regulated through activation of the (phosphoenolpyruvate-)glyoxylate shunt. The present work shows that detailed understanding of the genetic regulation of bacterial metabolism provides useful insights for manipulating the carbon flux in technical production processes.

A wealth of information is available on transcriptome responses in *Escherichia coli*, which are triggered through various stress conditions, including the limitation of energy and carbon sources. However, surprisingly little is known about the dynamic variation of gene expression under the physiological conditions that are required for technical production processes. Knowledge of this, however, is of great importance because *E. coli* has become the most widely used prokaryotic system for the production of heterologous proteins as well as for the industrial production of bacterial metabolites. Batch and fed-batch operations are the major cultivation strategies used for this purpose. For large-scale applications, fed-batch, high-cell-density *E. coli* K-12 derivative cultivation strategies have proven suitable for considerably increasing the volumetric productivity of these processes (37, 73). Irrespective of more sophisticated closed-loop strategies, fed-batch cultivations are

usually carried out with open-loop control via exponential or constant feeding. Exponential feeding maintains the specific growth rate at a constant level. The maximal biomass concentration that can be achieved with this strategy depends on sufficient oxygen supply and heat transfer capacities. At a constant feed rate, the specific growth rate gradually decreases due to declining carbon and energy source levels. The proceeding carbon limitation also leads to a range of serious starvation phenomena with manifold regulatory responses of the cells. These responses macroscopically manifest themselves in a loss of viability, such as was previously illustrated by Hewitt et al. (30).

Numerous experimental studies on transcription profiling have been carried out to characterize *E. coli* physiologically, in which a major focus has been put on high-cell-density cultivation with an exponential feed rate, growth on different substrates, and influence of regulatory proteins, diauxie, or starvation (a more detailed overview is given in Table S1 in the supplemental material). In spite of many physiological effects entailed by limited carbon concentrations, little is known about the thorough dynamics of regulatory events occurring in response to the proceeding carbon limitation during constant feeding conditions. Such knowledge, however, is essential for gaining a better understanding of the dynamic adaptation phenomena of *E. coli* during production processes. In the majority of investigations dealing with heterologous protein production, transcriptome data reflect a superposition of the effects of carbon limitation and foreign protein production. Therefore, valuable information on carbon limitation may be masked by

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other stress responses. A deeper insight into important regulation phenomena and the changes in metabolism in response to carbon limitation can therefore be gained only with investigations involving wild-type strains. Microarray data obtained from such cultivations should also be directly compared with transient variations of the metabolic fluxes to support hypotheses concerning the regulation of metabolic reactions.

One of the key regulation phenomena related to the onset of carbon limitation is what is known as the stringent response, which is mediated by guanosine 3',5'-bis(diphosphate) (ppGpp) (11), a nucleotide derivative that affects the affinity of RNA polymerase to different promoters and hence transcription (39). Elevated ppGpp concentrations lead to the reduced synthesis of ribosomal proteins, stable RNAs (tRNA and rRNA) and biosynthesis enzymes for fatty acids and lipids, and proteins involved in DNA replication. On the other hand, ppGpp is a positive regulator of the alternative sigma factor synthesis (σ^S , encoded by the *rpoS* gene) and, presumably as a secondary effect, of amino acid biosynthesis (1, 2, 11, 24, 38).

At low glucose concentrations, the limited amount of energy will be exploited as efficiently as possible by activating high-affinity glucose transport systems and by tapping alternative carbon sources (20, 31). This response, among others, is mediated by the *crp* modulon and occurs at the transition from exponential growth to the stationary phase (18). The *E. coli* fructose repressor, FruR (Cra), modulates the direction of the carbon flow by repressing the genes involved in fermentative carbon flow and by activating the enzymes involved in oxidative and gluconeogenic carbon flow (51). Submicromolar glucose concentrations (below 0.1 μM [0.02 mg liter⁻¹]) induce starvation responses that are mediated primarily by σ^S , which binds to RNA polymerase and leads to higher stress tolerance levels (20, 28), including resistance to stress factors such as H₂O₂, oxygen radicals, drought, acidic/basic pH, osmotic stress, and ethanol, as well as heat and cold (69).

However, little is known about the changes of the central carbon metabolism of *E. coli* K-12 wild-type cells grown under glucose-limiting conditions, nor is the chronological sequence of the aforementioned regulatory responses known in detail. One reason for this is the quick succession of the different glucose limitation stages in batch cultivations. Rapidly declining glucose concentrations lead to temporary alterations in the transport activities, which are difficult to investigate under these experimental conditions (21). Continuous cultivations involving wild-type and mutant *E. coli* strains, however, allowed the adjustment of micromolar glucose concentrations and dilution rates and were therefore assumed to generate an exactly defined physiological steady state that was stable over a long period of time. Nevertheless, subsequent experiments revealed that continuous-cultivation conditions led to changes on the transcriptome and proteome levels (34, 70). In addition, the long-term carbon limitation under continuous-culture conditions led to genomic mutations (45).

To shed further light on the sequence of bacterial responses induced at the transition from millimolar to submillimolar glucose concentrations, fed-batch cultivations of *E. coli* K-12 W3110 were performed at a constant feed rate. As shown previously, flux redistribution in the central carbon metabolism during carbon-limited growth results in a significantly lower biomass yield (27). The coordinate regulation of the expression

of many genes encoding enzymes of the central carbon metabolism was proposed as the most relevant process governing the observed behavior. In the current work, DNA microarrays were used to gain a holistic view of the dynamic changes occurring on the transcriptome level. A major focus was not only the time sequence of rearrangements of various cellular functions (transport, central carbon metabolism, growth, chemotaxis, and stress and starvation responses) but also the effects of these changes on the availability of resources for growth and maintenance. Thus, the work provides a comprehensive overview of the potentially critical responses that can be expected during carbon-limited biotechnological processes and which may have to be taken into account when rationally (i.e., through dynamic mathematical modeling) improving bacterial producer strains.

MATERIALS AND METHODS

Bacterial strain and cultivation. Three independent fed-batch cultivations of *E. coli* K-12 W3110 (DSM 5911) were carried out in a 30-liter bioreactor as described previously (27).

Transcriptome analysis using DNA microarrays. (i) **Printing of whole-genome DNA microarrays.** Whole-genome *E. coli* K-12 W3110 DNA microarrays were printed using the Microgrid II robot of Biorobotics (now Genomic Solutions, Huntingdon, United Kingdom) by spotting 4,608 oligonucleotides (50-mer, dissolved in buffer A; *E. coli* K-12 V2 Oligo set from MWG, Ebersberg, Germany) representing all *E. coli* K-12 open reading frames, 48 negative controls from *Arabidopsis*, and 47 duplicates on epoxy-coated glass slides (MWG). Tungsten microarray split pins (PT 3000; Point Technologies, Inc., CO) were used for spotting. For data analysis, a "gal file" was produced using the TAS Application Suite software version 2.6.0.2 from Genomic Solutions. Gal files allow the assignment of genes to the corresponding spots on the array. Further details on data analysis are given in "Data analysis" below.

(ii) **RNA isolation and precipitation.** The cell samples were collected directly into RNeasy Protect Bacteria reagent (Qiagen, Hilden, Germany) to avoid RNA degradation. The samples were centrifuged according to the manufacturer's protocol and frozen at -80°C until RNA isolation. Total RNA from 8.1×10^9 cells was isolated using the RNeasy kit (Qiagen). On-column DNase digestion was performed (RNase-free DNase set; Qiagen). RNA concentration and quality were assessed photometrically (Nanodrop ND 1000; NanoDrop Technologies, Inc., DE) by formaldehyde gel electrophoresis and bioanalyzer analysis (RNA 6000 Nano LabChip kit, Agilent Bioanalyzer 2100; Agilent Technologies, CA). Only RNA with a 260 nm/280 nm ratio of 1.8 to 2 as well as a 260 nm/230 nm ratio of >1.8 was used for precipitation. The RNA was precipitated and resolved at a final concentration of 6 $\mu\text{g}/\mu\text{l}$, which was required for reverse transcription and labeling with fluorescent dyes. The total RNA isolated was mixed with 1/10 volume of NaCl solution (3 M) and 2 volumes of ethanol (100%) and stored at -20°C overnight. The samples were subsequently centrifuged (14,000 rpm; 20,800 $\times g$), and the supernatant was discarded. The RNA was washed with ethanol (70%) and vacuum dried.

(iii) **Reverse transcription and labeling.** Concentrated total RNA was reverse transcribed using Superscript II RNase H reverse transcriptase (Invitrogen, Karlsruhe, Germany) and labeled with Cy3-dCTPs or Cy5-dCTPs. Reverse transcription was carried out as follows. Three microliters of random hexanucleotide primers (6 N) and 16.5 μl of precipitated RNA (total, 100 μg) were mixed and incubated at 65°C for 10 min. The reaction mixture was then incubated at room temperature for a further 10 min and on ice for 2 min. Eight microliters of 5 $\times$ RT reaction buffer (Superscript II kit; Invitrogen), 4 μl of deoxynucleoside triphosphate master mix (dATP, 5 mM; dGTP, 5 mM; dTTP, 5 mM; dCTP, 2 mM), 4 μl each of Cy3-dCTP and Cy5-dCTP, 4 μl of dithiothreitol (0.1 M) (Superscript II kit; Invitrogen) and 1.5 μl of Superscript II (200 U) (Invitrogen) were added. This mixture was incubated for 2 h at 42°C . The reaction was stopped by adding 10 μl of a sodium hydroxide solution (1 M) and heating to 65°C for 10 min. The solution was neutralized by adding 10 μl of hydrochloric acid (1 M), and then 200 μl of 1 $\times$ Tris-EDTA buffer was added and the mixture purified using the PCR purification kit as recommended by the manufacturer (Qiagen). The cDNA concentration was determined photometrically (Nanodrop ND 1000; NanoDrop Technologies, Inc.), and on average, 3,400 ng of each labeled cDNA of the corresponding point in time (T, versus the reference [R]) were mixed in one Eppendorf cup and vacuum dried at 30°C until nearly dry. The

FIG. 1. Glucose-limited fed-batch cultivation of *E. coli* K-12 W3110 with a constant feed rate and sampling for transcriptome analysis. The vertical line at $t = 0$ indicates glucose limitation (as judged from the dissolved oxygen concentration time course [data not shown]). The concentrations of biomass (■), glucose (▼), and acetate (□) are given, as well as the time course of the specific growth rate (μ) (broken line). Arrows above the graph indicate the times when the samples were removed for microarray analysis (R, reference; T1 to T8, time series samples).

cDNA was redissolved in 65 μ l prewarmed (42°C) hybridization buffer (MWG) and stored for subsequent use, either on ice for less than 1 h or at -20°C when stored overnight. The reverse-transcribed probes were hybridized on the arrays as described in "Experimental design" below.

(iv) **Experimental design.** Hybridization experiments included a reference sample that was common to all arrays (14). A sample of the unlimited growth phase from the batch phase of the cultivation process was chosen (see Fig. 1 for details) as the reference (R). This was hybridized on an array together with the sample of interest (T1 to T8) while different labeling agents were used (Cy3 and Cy5). This allowed the direct comparison of the transcripts from a time point of the series taken during fed-batch cultivation with the unlimited reference sample. All samples were used in duplicate, and the experiments were performed as dye swap experiments (14). The first four samples of the time series (T1 to T4) were taken from three individual fed-batch cultivations (three biological replicates); the subsequent four samples (T5 to T8) were taken from only two individual fed-batch cultivations. On each array, the genes were spotted in duplicate. Therefore, 12 replicates (duplicates on dye swaps for three cultivations) were analyzed for T1 to T4, and eight replicates (duplicates on dye swaps for two cultivations) were analyzed for T5 to T8. Time and biological replicates were treated as two different experimental dimensions. Therefore, the time stages of one biological replicate were not considered independent. A high correlation was observed for the dye swap pairs (with average Pearson's correlation coefficients of 0.89 ± 0.06 for the reference samples and 0.87 ± 0.08 for the different points in time) as well as for biological replicates (with average Pearson's correlation coefficients of 0.86 ± 0.04 for the red channel [Cy5] and 0.80 ± 0.09 for the green channel [Cy3]). This high positive linear correlation is equivalent to the high reproducibility of the biological replicates and the dye swap pairs.

(v) **Hybridization.** The slides were hybridized in the fully automatic HS400 hybridization station (Tecan Deutschland GmbH, Crailsheim, Germany). Labeled cDNA samples were prepared by heating them to 95°C for 3 min. The hybridization lasted for 12 h at 42°C ; $6\times$ SSPE ($20\times$ stock SSPE was $175.3 \text{ g} \cdot \text{liter}^{-1} \text{ NaCl}$, $27.6 \text{ g} \cdot \text{liter}^{-1} \text{ NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and $7.4 \text{ g} \cdot \text{liter}^{-1} \text{ Na}_2\text{EDTA}$) was used for prewashing, and washing buffer 1 ($17.6 \text{ g} \cdot \text{liter}^{-1} \text{ NaCl}$, $8.8 \text{ g} \cdot \text{liter}^{-1}$ sodium citrate, and $1 \text{ g} \cdot \text{liter}^{-1}$ sodium dodecyl sulfate) and washing buffer 2 ($0.88 \text{ g} \cdot \text{liter}^{-1} \text{ NaCl}$ and $4.4 \text{ g} \cdot \text{liter}^{-1}$ sodium citrate) were used for washing the hybridized slides, which were subsequently dried with nitrogen gas for 2 minutes. The following hybridization protocol was used: (i) washing at 42°C (once for 30 s, 30-s hold, $6\times$ SSPE, (ii) sample application at 42°C , (iii) hybridization at 42°C (shaking frequency medium, 12 h), (iv) washing at 23°C (twice for 1 min, 30-s hold, washing buffer 1), (v) washing at 23°C (twice for 1 min, 30-s hold, washing buffer 2), and (vi) drying (30 s, 2 min, N_2).

(vi) **Scanning process.** The completely dried DNA microarrays were scanned using the ScanArray Express microarray scanner (Perkin-Elmer, MA) (resolution, $5 \mu\text{m}$; PMT setting, 70). Pictures of both channels were saved as 16-bit TIFF files.

(vii) **Data analysis.** Raw data were created by analyzing 16-bit TIFF files with the ScanArray Express imaging software (microarray analysis system, version

3.0.0.0016; Perkin-Elmer, MA) (adaptive threshold method, total normalization method). The data were statistically analyzed using the software R (52) and the Limma package (58). Limma applies linear models to analyze designed experiments and to identify differentially expressed genes. An empirical Bayes method was chosen to take into account that in the classical t test, small variance values of the gene of interest tend to lead to high t test statistics and therefore, erroneously, to a higher probability of significant differential expression. The parametric empirical Bayes approach (38a) involves a so-called "penalty value" which is estimated from the mean and standard deviation of the random sample variance. This leads to an adjusted test statistic and therefore to more reliable results. Furthermore, Smyth's empirical Bayes approach (58), which includes the variance of all genes to calculate single t test statistics, was used to obtain reproducible data. Empirical Bayes and other shrinkage methods are used to borrow information across genes, making the analyses stable even for experiments with small number of arrays (59, 60; cited in reference 58). The data was normalized "print-tip-loess" (61). Spots which were classified as not found by the image analysis software were downweighted to 10%.

As thousands of hypotheses can be tested with DNA microarrays, the chance to make an α error increases with the number of hypotheses. Therefore, multiple-hypothesis testing (15a) was performed. The false discovery rate (FDR) was selected, which takes into account an expected contingent on errors of genes being identified as differentially expressed. Multiple-hypothesis testing correction was performed by the method of Benjamini and Hochberg (5), which controls the FDR. The quotient between expected false-positive genes and all differentially expressed genes allows an adjusted P value to be calculated, which can be used to make predictions about differential gene expression. Genes were regarded as differentially expressed when the adjusted P values were <0.05 , therefore nominally controlling the expected FDR to less than 0.05. The reproducibility of dye swap experiments as well as biological replicates was estimated by calculating Pearson's correlation coefficients.

Metabolic flux analysis. Metabolic flux analyses were performed by stoichiometric metabolite balancing (43, 64) of five independent fed-batch cultivations using the Insilico Discovery 1.2 software (Insilico Biotechnology AG, Stuttgart, Germany) as previously published (27).

Microarray data accession number. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE10307 (3, 16).

RESULTS AND DISCUSSION

Fed-batch cultivations. Three independent fed-batch cultivation experiments using a constant feed rate were performed to study the global physiological response of *E. coli* to gradually decreasing glucose concentrations. Technical details of these cultivations were previously published by Hardiman et al. (27). The extracellular glucose, acetate, and biomass concentrations are depicted in Fig. 1. In the batch phase ($t < 0$) (Fig. 1), acetate accumulated during the consumption of glucose (metabolic overflow). The fed-batch phase was started upon glucose limitation at the end of the batch phase ($t = 0$) (Fig. 1) (27). The accumulated extracellular acetate was consumed at the beginning of the subsequent glucose-limited fed-batch phase ($t > 0$) (Fig. 1). The constant-feeding strategy used led to a gradual decrease in the specific growth rate, μ (Fig. 1). The qualitative time course of the extracellular glucose concentration, obtained using Monod-type kinetics, and the experimentally determined growth rate under the assumption of constant substrate affinity (Monod constant $[K_S] = 0.05 \text{ g l}^{-1}$) (72) throughout the cultivation process are also given in reference 27. The concentration was below $120 \text{ mg liter}^{-1}$ when the feed was started and decreased to at least 6 mg liter^{-1} (27). However, particular rearrangements in the transport systems, which will be illustrated below (see "Transport systems" under "Global transcription analysis"), led to changes in substrate affinity. The extracellular glucose concentration was therefore expected to be considerably lower during fed-batch cultivation.

The biomass yield, $Y_{X/S}$, decreased by 20% during fed-batch growth, while the maintenance energy coefficient, m_s , showed a steep increase (27).

Experimental design. A time series of a global transcription analysis was carried out for three independent glucose-limited fed-batch cultivations. cDNA samples were generated from the phase of unrestricted growth (reference sample R) (Fig. 1, batch) as well as from the carbon-limited growth phase (samples T1 to T8) (Fig. 1, fed batch). These were hybridized to whole-genome microarrays of *E. coli* K-12, in which each sample was cohybridized with a reference state sample (Fig. 1). The individual samples corresponded to the following process/physiological conditions: T1, glucose limitation (glucose concentration of <0.05 g liter $^{-1}$); T2, glucose limitation, acetate concentration of 0.35 g liter $^{-1}$; T3, glucose limitation, 30 min after depletion of extracellular acetate; T4, glucose limitation, 50 min after depletion of extracellular acetate; T5 to T7, glucose limitation, 1-hour intervals corresponding to specific growth rates of 0.16 h $^{-1}$, 0.13 h $^{-1}$, and 0.11 h $^{-1}$; and T8, glucose limitation, after 7 hours of fed-batch growth ($\mu = 0.08$ h $^{-1}$).

Global transcription analysis. The statistical analysis of the data revealed significant differences between the time series samples (T1 to T8) and the unlimited-growth reference sample (R). Genes were regarded as differentially expressed when their Benjamini-Hochberg adjusted P value from an empirical Bayes moderated t test was <0.05 . The calculated average $\log_2$ ratios of all differentially expressed genes ranged between -2.6 and 3.2 . These values corresponded to an up to sixfold down-regulation and an up to ninefold upregulation between T1 to T8 and the reference sample. The 2σ intervals (twice the standard deviation) of the $\log_2$ ratios ranged between 0.2 and 1.3 . Under the assumption that the $\log_2$ ratios were normally distributed, 95% of these values were within the denoted range (average $\log_2$ ratios $\pm 2\sigma$). Quantitative PCR was performed for selected genes (see GEO [http://www.ncbi.nlm.nih.gov/geo/]). The results were in good agreement with the microarray data.

The transcription profiles of 960 genes changed significantly at at least one point in time compared to the reference (see GEO [http://www.ncbi.nlm.nih.gov/geo/]). A total of 595 of these transcripts could be assigned to the physiological functions of the central carbon metabolism (glycolysis, Entner-Doudoroff pathway, pentose phosphate pathway (PPP), tricarboxylic acid [TCA] pathway, glyoxylate shunt (GS), and respiration (58 transcripts), transport (121 transcripts), anabolism (128 transcripts), catabolism and macromolecular degradation (51 transcripts), protein biosynthesis (81 transcripts), cell division (16 transcripts), stress response (33 transcripts), flagellar and chemotaxis system (17 transcripts), regulation (48 transcripts), and other proteins (44 transcripts). The remaining 365 transcripts coded for hypothetical proteins or for proteins of unknown function. A general overview of the observed behavior is given in Fig. 2, which also summarizes the information discussed in the following sections.

(i) Transport systems. Existing transport systems involved in glucose uptake in *E. coli* have been reviewed by several teams of scientists (19, 26, 49). With respect to their affinity for glucose, these transporters are generally classified as high, medium, or low. Moreover, the transporters are sometimes also

classified according to their specific location (inner and/or outer membrane) or according to their specific mechanism (phosphotransferase system [PTS]; ABC transport system; or uni-, sym-, or antiporter system). Transport systems of high affinity (ABC transport systems with low K_m values of <10 μ M), moderate affinity (phosphoenolpyruvate [pep]-dependent PTSs with K_m values of 10 to $1,000$ μ M), and lower affinity (e.g., symporters with low K_m values ranging between 0.1 and 10 mM) are found in the inner bacterial membrane (18, 49). Several porins, with either high or low affinities, are located in the outer cell membrane (18).

Ferenci (18) found that under glucose-limited steady-state conditions, *E. coli* expresses predominantly the high-affinity transporters of the outer (e.g., porin LamB) and inner (e.g., galactose ABC transporter encoded by *mglBAC*) membranes. Such chemostat cultivations are of limited use for production purposes. Fed-batch strategies (performed at either constant or exponential feed rates) are often the method of choice. Since both strategies are based on limiting bacterial growth by varying the concentration of the substrate, it was important to further investigate whether a constant feed rate, which consequently results in decreasing glucose concentrations, would lead to transportation behavior that was similar to that of chemostat cultures.

The observed dynamic response caused by this feeding strategy is summarized in Fig. 3. The majority of transport systems involved in glucose uptake were differentially expressed during the fed-batch cultivation process. This was particularly so for the porin LamB (encoded by *lamB*) (Fig. 3a and b), which is a high-affinity glucose transporter located in the outer membrane. This particular porin was expressed predominantly during the first 2 hours of glucose limitation, whereas the two other outer membrane porins, encoded by the *ompF* and *ompC* genes, were not affected (Fig. 3b).

The antiport system GalP (*galP*) and two genes of the galactose ABC transporter (*mglA* and *mglB*), which transport glucose with high affinity (20), were also expressed predominantly during the first 2 hours of glucose limitation (Fig. 3a and b). The differential expression of *mglB* was confirmed by real-time PCR (see GEO [http://www.ncbi.nlm.nih.gov/geo/]). The mannose PTS (encoded by *manXYZ*), which transports glucose with moderate affinity (48), was preferentially expressed between time points T1 and T6. It can be further assumed that the glucose PTS was active during the entire glucose limitation period, since none of the respective mRNA levels (*crr*, encoding EI^IA^{Glc}, and *ptsG*, encoding EI^IBC^{Glc}) decreased at all. It is interesting to note that the aforementioned differentially expressed ABC transport systems and the GalP antiport system are unable to directly phosphorylate glucose during transport (Fig. 3b). In accordance, the glucokinase-encoding gene (glucokinase is a protein that converts glucose to glucose 6-phosphate [g6p] [41]) was differentially expressed between T2 and T4.

The topology of the stimulus-response cascade of the glucose transport system, exhibiting various feed-forward loops and involving the alarmone cyclic AMP (cAMP) as a major internal signal, can be deduced from the observed time course of transcript levels (Fig. 3b and c). In previous studies using the same set of cultivations (27), we were able to demonstrate that cAMP was produced continuously when the external glucose

FIG. 2. Global regulation of the *E. coli* K-12 W3110 metabolism during carbon-limited growth, derived from a genome-wide transcriptome and metabolic-flux analysis. The regulatory processes that are most relevant for utilization of available intra- and extracellular resources are proposed. mRNA/flux levels: +, increase; -, decrease; =, invariable. Short dashed lines, transcriptional regulation; long dashed lines, signaling processes (regulation of protein activities: arrowhead, positive; blunt end, negative). (I) A cluster of high-affinity transporters is synthesized (*mglBA*, *galP*, and *lamB*), while the activity of medium-affinity transporters is maintained. This is due mainly to their regulation by the Crp-cAMP complex but also to the effect of the transcriptional regulatory proteins MalT, YeeI, and Mlc (Fig. 3b and c). The glucose flux entering the cell is directed via transporters that do not use pep for phosphorylation. This preserves the pool of this metabolite (homeostasis) and affects the EIIA^{Glc}-P-dependent activation of cAMP synthesis through the enzyme adenylate cyclase (CyaA). (II) These transport systems in particular depend on a membrane proton gradient for proper function. The expression of the proton gradient-dependent chemotaxis system is reduced, thereby enabling the transport system to effectively utilize the energy available. (III) The flux through the upper part of glycolysis is favored, whereas the flux through the PPP is minimized, which is most likely due to the reduced synthesis of *gnd* mRNA. The flux entering the PPP is used for biosynthesis at the expense of the reflux into the glycolysis pathway, which might be regulated by the RpiA/Rpe split ratio. The reaction rates in the lower glycolysis decrease due to decreasing mRNA levels (*cra* modulon; signaling through *fbp*), thereby providing a sufficient, though minimal, efflux into the PPP. The carbon flux entering the TCA cycle (influx is enhanced via *gltA* expression) is split into the GS, the PEP-GS, and the full TCA cycle. The GS and PEP-GS provide a better pep, pyruvate (pyr), and oac precursor supply. It is assumed that the global regulation via the *crp* and *cra* modulons is the most relevant in this respect. (IV) Cellular growth is regulated predominantly by the stringent response (alarmone ppGpp, *relA/spoT* modulon). (V) No extensive induction of the general *rpoS*-dependent response could be observed (opposing regulation via the *crp* and *relA/spoT* modulons). It is expected that slow substrate concentration changes do not trigger a strong starvation response. However, other stress responses were detected. Details are presented in the text.

concentration declined from 0.7 mM to at least micromolar concentrations at time point T8. All glucose transport systems identified are positively regulated by the Crp-cAMP complex (Fig. 3b and c) (9, 68). Although the *crp* gene was not differentially expressed, the genes encoding the regulatory proteins MalT and YeeI (Fig. 3c) were. MalT generally regulates the expression of transport systems (15) and underwent active transcription (Fig. 3b and c). The Crp-cAMP complex enhances the expression of this gene (Fig. 3c). Finally, MalT positively regulates the expression of transport proteins such as MalK, LamB, and MalM (9). LamB transcripts were differentially expressed (Fig. 3b). Mlc regulates several genes that are involved in glycolysis and glucose uptake. In particular, it represses genes of the glucose-specific PTS (*ptsG*, *ptsHI-crr*), the regulatory protein MalT, and the *manXYZ* operon (8, 25, 48) (Fig. 3b and c). The repressor protein Mlc interacts with the dephosphorylated PtsG protein (which is the EIIB^{Glc} subunit of the glucose-specific PTS) under nonlimited growth conditions and can repress MalT. In the present investigation, the glucose concentration seemed not to be low enough to lead to the inactivation of MalT (Fig. 3b and c). Thus, the repressor remained inactive, which might explain the absence of fur-

ther changes in the glucose-specific PTS-transcript levels during carbon-limited conditions at fed-batch cultivation. Recently, Becker et al. (4) identified the YeeI protein (now designated MtfA [Mlc titration factor]), which, if present, interacts with Mlc, thereby leading to its inactivation. Elevated levels of YeeI RNA transcripts were observed over the whole period analyzed (Fig. 3c). Therefore, Mlc seems to be under the dual control of YeeI and phosphorylated PtsG, two proteins that ensure that Mlc is inactivated under the growth conditions examined.

It seems that the inferred genetic regulatory network (Fig. 3b and c) provokes the preferential expression of the high-affinity transporters GalP and Mgl, while at the same time, medium-affinity transporters such as the glucose-dependent PTS remain active (Fig. 2). These physiological refinements are important for sustaining the maximal influx of glucose. In our investigations, a gradually decreasing glucose flux was observed during the ongoing fed-batch process (see "Central carbon metabolism" below). This phenomenon is due to the constant feed rate chosen to technically control the glucose uptake rate.

FIG. 3. Dynamic changes in transporter mRNA levels and their regulation in *E. coli* K-12 W3110 during glucose-limited fed-batch growth with a constant feed rate. (a) Sugar transport systems. (b) Regulation of transporter gene expression. (c) Expression of proteins relevant for the regulation of transporters. The time courses of the transcript levels are given for samples T1 to T8 relative to the reference sample in the batch phase (R) (Fig. 1). Green, mRNA level lower than in the reference state. Red, higher mRNA level. Statistical significance ($P \leq 0.05$) is indicated by asterisks. Glc, glucose; P, phosphoryl group. Node symbols (states): rectangle, gene; parallelogram, RNA; rounded rectangle, protein; black rounded rectangle, regulator protein; gray rounded rectangle, protein with differentially expressed mRNA. Arrow symbols: solid line, regulatory interaction; dash-dot-dot-dash line, transcription; dash-dot-dash-dot line, translation. Arrowhead symbols: filled arrow, transformation; blunt-end arrow, inhibition or repression; open arrow, activation; filled arrow with crossbar: transport.

The specific refinement of the transport system to rather high-affinity systems might also be of relevance in terms of homeostasis. The limited availability of glucose also reduces the metabolic flux through glycolysis (see below). Consequently, the pools of crucial metabolites such as pep, which plays an intermediate role between energy supply and anabolism, might drop to critically low concentrations. The refined (high-affinity) system consists predominantly of ABC or sym- or antiporter proteins, which are altogether ATP dependent, rather than of pep-dependent PTSs of medium affinity (Fig. 3a). It can therefore be assumed that the glucose flux is directed via ATP-dependent transporter systems and that pep serves mainly as precursor for anabolic purposes (Fig. 2). Moreover, the pep pool is important for the Crp-cAMP complex-mediated regulation, as it determines the phosphorylation state of the PTS (Fig. 3a). At high pep levels, cAMP synthesis, which is catalyzed by adenylate cyclase (CyaA), is activated via the phosphorylated protein $\text{EIIC}^{\text{Glc}}\sim\text{P}$ (Fig. 3a). As previously described by Hardiman et al. (27), a maintained pep pool, accompanied by the low flux via pep-dependent transporters, might thus lead to continuous cAMP synthesis (and export) during fed-batch growth (Fig. 2).

In addition to the refined glucose uptake system, the expression of proteins that transport other substances indicates the bacteria's reorganization abilities, which adapt the *E. coli* metabolism to altered environmental conditions: in total, at least 121 transcripts of proteins involved in transport processes of sugars, amino acids, fatty acids and metals were differentially expressed in response to declining glucose concentrations (see the supplemental material).

(ii) Central carbon metabolism. As mentioned above, the dynamic behavior of the central carbon metabolism of *E. coli* K-12 and its regulation under glucose-limiting conditions are not yet understood in detail. Metabolic flux analyses of *Escherichia coli* K-12 W3110, previously undertaken with the same set of fed-batch experiments, showed that glycolysis and PPP fluxes decreased strongly, while the fluxes in the TCA cycle remained constant (27). This behavior was attributed to the regulation of the expression of many genes of the central carbon metabolism by the *crp*, *cra*, and *relA/spoT* modulons. In the present investigation, the time courses of the transcript levels could be determined for the genes of the central carbon metabolism (Fig. 2 and 4). These were compared with the metabolic fluxes and provided substantial evidence for the regulatory model structure previously suggested by Hardiman et al. (27).

(a) Glycolysis (*Embden-Meyerhof-Parnas [EMP]*) pathway and PPP. Glucose influx decreased dramatically during fed-batch growth as a result of constant glucose feeding (27). In order to determine the changes in metabolic fluxes that could not be attributed to the general flux decrease in the network, the glucose influx was taken as reference value and set to 100% at each point in time (Fig. 4). In response to the limited carbon supply, the expression of the majority of the glycolysis transcripts decreased considerably, whereas the number of isoenzyme transcripts increased (*pfkA*, *fbaB*, and *gpmB*) (Fig. 4). Apparently, enzyme levels are downregulated according to the decreased flux caused by constant feeding. Considering the reduction of the reaction rates, $r_i = r_{\max,i} \cdot f(c_j)$, it can be assumed that the downregulation of the enzyme levels (i.e.,

FIG. 4. Time series of DNA microarray and metabolic-flux analyses of the central carbon metabolism in *E. coli* K-12 W3110 during glucose-limited fed-batch growth with a constant feed rate. The time courses of the transcript levels are given for samples T1 to T8 relative to the reference sample in the batch phase (R) (Fig. 1). Green, mRNA level lower than in the reference state. Red, higher mRNA level. Statistical significance ($P \leq 0.05$) is indicated by asterisks. The metabolic fluxes are given for the -0.3 h (batch), 3.9 h, and 7.7 h (fed batch) (Fig. 1). Fluxes are mean values from the stoichiometric metabolite balancing of five independent cultivations and are given as molar percentages of the glucose influx. Notation is according to reference 27.

maximal reaction rates, $r_{\max}$) leads to constant metabolite concentrations, c_j (homeostasis). The reactions catalyzed by the PTS, 6-phosphofructokinase (PfkA), pyruvate kinase (PykF), and pyruvate dehydrogenase have high flux control coefficients

(12). Therefore, it can be expected that the respective enzyme levels are regulated. It is known that most of the glycolysis genes that are less transcribed (Fig. 4) are repressed by the global regulator protein Cra; these include *pfkA*, *fbaA*, *pgk*,

pykF, *gapA*, and *eno* (see the EcoCyc database) (33, 56). Moreover, the enzymes PfkA and PykF were less active during glucose limitation (57). The Cra protein is inactivated by the metabolite fructose 1,6-bisphosphate (fbp) (50), whose level decreases during carbon-limited growth and signals the absence of glucose (27). This leads to the Cra-dependent repression of glycolysis genes and also to the flux decrease observed (Fig. 4). It is therefore proposed that the observed behavior is regulated by the *cra* modulon (Fig. 2). This results in sufficient, though minimal, efflux into the PPP, thereby maintaining the cell's supply of biosynthesis precursors.

However, the flux into the PPP decreased more than the fluxes into the glycolysis pathway (EMP/PPP split ratio) (Fig. 4). In accordance, the flux fraction via the upper part of glycolysis (from g6p to glyceraldehyde 3-phosphate [gap]) increased during carbon-limited growth at the expense of the flux via the PPP (g6p to fructose 6-phosphate and gap) (Fig. 4). It is expected that the PPP flux is controlled via regulation of a reaction catalyzed by 6-phosphogluconate dehydrogenase (Gnd), i.e., one of the two irreversible reaction in the PPP, which can operate as control point between the oxidative and the nonoxidative branches of the PPP (63). It is already known that the amount of Gnd protein is determined primarily by the rate of transcription initiation (46, 71). It is assumed that the amount of Gnd correlates with the growth rate (46, 71). However, this has so far been shown only for balanced-growth experiments with, e.g., acetate or glucose as carbon and energy source (46, 71). During fed-batch cultivation, the transcript level of *gnd* decreased considerably (Fig. 4), which substantiates the assumption of its correlation with the specific growth rate. The underlying regulatory mechanism has, however, not yet been clarified. Even more difficult is the situation with the second irreversible reaction catalyzed by the G6P-1-dehydrogenase (Zwf). Rowley and colleagues found that the *zwf* transcription rate also varied with the type of substrate used (53). In contrast, the gene was not differentially expressed during fed-batch growth. The lower *gnd* mRNA levels are in accordance with an increase in the flux fraction via the upper glycolysis part (Fig. 4). It is therefore assumed that the negative regulation of *gnd* transcription is a key parameter for understanding the observed differences in the EMP/PPP split ratio (node g6p, Fig. 4) (Fig. 2). In addition to this, there are also several factors that fine-tune the activities of the PPP enzymes, e.g., the NADP⁺/NADPH ratio and the fbp, g6p, and ribulose 5-phosphate (ribu5p) concentrations (33, 54). The regulation of PPP enzymes is expected to minimize the oxidation rate of the substrate and reduce the efflux into biosyntheses to a minimum.

Moreover, higher *rpiA* and lower *rpe* mRNA levels could be detected during fed-batch growth (Fig. 2 and 4). The corresponding fluxes were in accordance with the gene expression levels (Fig. 4): the RpiA flux (from ribu5p to xylulose 5-phosphate; enzyme, ribose 5-phosphate isomerase A) decreased less than the Rpe flux (ribu5p to ribose 5-phosphate; enzyme, ribulose phosphate 3-epimerase). The splitting of the flux at the node ribu5p (RpiA/Rpe) is also reflected in the ratio of the efflux into biosyntheses (ribose 5-phosphate to 5-phosphoribosyl 1-pyrophosphate) and the flux reentering the EMP (xylulose 5-phosphate to fructose 6-phosphate and gap), which increases during carbon-limited growth (0.67, 0.70, and 0.73)

(Fig. 4). In other words, the flux that enters the PPP is preferentially directed toward biosynthesis. Although it is assumed that RpiA and Rpe protein level regulation is unimportant for the control of the overall flux through the central carbon metabolism (12, 35), the regulation of the RpiA/Rpe split ratio might fine-tune the efflux into the nucleotide, histidine, and tryptophan biosynthesis pathways (Fig. 2). Simulation of the glycolysis pathway and the PPP using the model of Chassagnole et al. (12) and varying the $r_{\max}$ values of the two reactions confirmed this assumption and suggested that the high *rpiA* mRNA level (Fig. 4) could play a major role (data not shown). However, little is known about the regulation of the respective genes (23, 33, 63).

(b) *TCA cycle and GS*. Previous investigations found that the reaction rates in the TCA cycle remained constant during glucose-limited fed-batch growth, and this was regarded as a major reason for the substantial decrease in the biomass yield (27). Hardiman et al. (27) integrated the existing knowledge about the modular regulation of the enzymes of the central carbon metabolism into a comprehensive and global structure. Within this systems-oriented picture, it is assumed that the Crp-cAMP complex (with a strong increase in cAMP) activates the expression of TCA cycle genes in a coordinated manner (27). It is supposed further that the Cra regulator protein activates the transcription of GS genes. These hypotheses are supported through findings obtained with microarray analyses (Fig. 2 and 4). The relatively high influx into the TCA cycle is most likely due to a higher GltA enzyme level, since the GltA mRNA level is much higher (Fig. 4). The regulation of the GltA protein level for the major control of the flux into the TCA cycle has also been predicted from thermodynamic analyses (35). In general, the expression of TCA cycle genes was considerably higher during fed-batch conditions, in particular for those genes whose expression is positively regulated by the Crp-cAMP complex (*gltA*, *acnB*, *sucABCD*, and *sdhCDAB*) (Fig. 4). A considerable increase in the expression of GS genes (*aceB* and *aceA*) was also found (Fig. 4), suggesting that the products of the *gltA*, *acnB*, and *sdhCDAB* genes (Fig. 4) are also involved in the GS. It must therefore be assumed that a large fraction of the flux entering the TCA cycle must be directed into the GS. The pep-glyoxylate cycle (PEP-GS) is another alternative cycle for substrate oxidation (22). This cycle involves the flux through the GS and the cycling of oxaloacetate (oac) to pep, catalyzed by pep carboxykinase (PckA). Although the *pckA* gene was not differentially expressed in microarray experiments, quantitative PCR analysis nevertheless showed that the gene transcript was more abundant during fed-batch growth (<http://www.ncbi.nlm.nih.gov/geo/>). In *E. coli* MG1655, the TCA/GS/PEP-GS flux ratio was determined as 1.1:1.5:1.0 at $\mu = 0.12 \text{ h}^{-1}$ (22) or 0.9:1.1:0.2 in a derivative of MG1655 (42). These data suggest that the close relative *E. coli* K-12 W3110 also uses these cycles. Considering the cycles' functions, it seems likely that the maintenance of the oac, pep, and pyruvate precursor pools is a major goal of the behavior observed, besides supplying the cells with sufficient metabolic energy. The importance of the pep pool for the import of glucose and the synthesis of the signaling molecule cAMP was highlighted above (see "Transport systems"). Another benefit for the cells could be seen in the reduced oxidation of the substrate and the reduced production of NADPH (via isoci-

TABLE 1. Flagellar/chemotaxis system

Category and gene	Gene product	Expression ^a at:							
		T1	T2	T3	T4	T5	T6	T7	T8
Early class									
<i>flhD</i>	Transcriptional dual regulator SU	0.18	-0.20	-0.39	-0.05	-0.12	-0.33	-0.37	-0.50
Middle class									
<i>fliA</i>	Sigma 28	0.10	-0.26	-0.29	-0.64	-0.55	-0.68	-1.00	-2.42
<i>ycgR</i>	Involved in flagellar motility	0.08	0.02	-0.74	0.15	0.06	0.06	0.22	0.05
<i>flgB</i>	Basal body rod protein	0.07	-0.10	-0.40	-0.29	0.07	-0.20	-0.45	-1.32
<i>flgD</i>	Initiation of hook assembly	-0.22	-0.25	-0.35	-0.59	-0.41	-0.54	-0.98	-1.44
<i>flgE</i>	Flagellar hook protein	0.20	-0.56	-0.68	-0.52	-0.27	-0.58	-0.87	-1.96
<i>flgC</i>	Basal body rod protein	-0.09	-0.47	-0.40	-0.35	-0.50	-0.63	-0.99	-2.04
<i>flgA</i>	Flagellar biosynthesis	-0.08	-0.12	0.03	-0.31	0.09	0.36	-0.05	-0.71
<i>flgJ</i>	Flagellum-specific muramidase	-0.03	-0.45	-0.63	-0.52	-0.08	-0.08	-0.30	-0.61
<i>flgG</i>	Basal body rod protein	-0.09	-0.43	-0.95	-0.36	-0.02	-0.41	-0.41	-0.98
<i>flgH</i>	Flagellar L-ring protein	-0.09	-0.40	-0.48	-0.03	0.33	-0.03	-0.17	-0.59
<i>flgK</i>	Hook filament junction protein 1	-0.08	-0.44	-0.64	0.06	-0.04	-0.31	-0.39	-0.77
<i>fliD</i>	Flagellar cap protein	0.09	-0.08	-0.39	-0.25	-0.14	-0.15	-0.24	-0.25
<i>fliS</i>	Flagellum biosynthesis protein	-0.04	-0.24	-0.59	-0.63	-0.34	-0.30	-0.63	-0.90
<i>fliM</i>	Motor switch protein	-0.01	-0.32	-0.54	-0.49	-0.33	0.18	-0.29	-1.04
<i>fliE</i>	Basal body protein	-0.36	-0.81	-0.51	-0.84	-0.47	-0.24	-0.39	-1.20
Late class									
<i>motA</i>	Flagellar motor complex component	-0.20	-0.19	-0.62	-0.22	-0.21	-0.83	-0.23	-0.27
<i>cheR</i>	Chemotaxis protein methyltransferase	0.09	-0.05	-0.42	-0.40	-0.20	0.12	-0.02	0.11
Other									
<i>fliA</i>	Flagellar system protein	0.03	0.06	0.07	0.47	0.08	0.31	0.21	0.12
Associated regulatory proteins									
<i>csrA</i>	Carbon storage regulator	0.02	-0.48	-0.29	-0.31	-0.50	-0.49	-0.07	-0.15
<i>hns</i>	Transcriptional dual regulator	0.13	0.32	0.42	0.63	0.09	1.21	0.28	0.06
<i>lraA</i>	Transcriptional repressor	-0.07	0.09	0.36	0.31	0.16	0.32	0.42	0.33
<i>ygiX</i>	Transcriptional activator QseB	-0.20	-0.17	0.08	-0.41	-0.15	-0.26	-0.09	-0.03

^a Coefficients of the linear model. Underlining indicates significantly differential expression.

trate dehydrogenase [IcdA]), which is not needed in large amounts due to the slower growth of the cells.

(iii) **Chemotaxis and flagellar system.** Chemotaxis is a phenomenon involving bacterial movement along the concentration gradients of certain chemicals (see reference 17 for further details). Chemotaxis helps bacteria to detect food sources by swimming toward the highest concentration of food molecules (for example, glucose). *E. coli* has flagella that rotate principally in two opposing ways, enabling the bacteria to change directions. More than 50 genes are required for the synthesis and function of the *E. coli* flagellar and chemotaxis system (13). These genes, which belong to 17 operons, constitute a regulon within which the operons are grouped into three temporally regulated, hierarchically organized transcriptional classes: early, middle, and late.

The use of laboratory-scale bioreactors guarantees homogeneously mixed cultivation media at any time. Therefore, different regulatory responses due to local substrate gradients seem to be irrelevant for these bioprocesses. On the other hand, the chosen feeding strategy leads inevitably to the above-mentioned time profile of limiting glucose concentration.

Therefore, it was investigated whether this feeding strategy might lead to a global stimulus of the chemotaxis response.

As shown in Table 1, nearly 50% of all genes known to be involved in the chemotaxis and flagellar system were affected during the entire cultivation process. The chemotaxis system seemed to be active during the nonlimiting exponential growth phase. Over time, the majority of chemotaxis genes were transcribed to a lesser degree (Table 1). The number of transcripts of one of these genes, the dual regulator *flhD*, which is responsible for initiating the chemotaxis system, was reduced as early as T2 and subsequently followed by middle and late class genes (Table 1). It can therefore be assumed that the entire functional flagellar system is displaced from the cell under enhanced carbon limitation (e.g., at T3, when external acetate has been consumed).

As previously shown by Hardiman et al. (27), the intracellular cAMP levels rose considerably at the beginning of glucose limitation. This coincided with the time when the transcript levels of the chemotaxis genes were reduced (Table 1). The specific role of the alarmone cAMP in bacterial chemotaxis has been under debate for many years. While cAMP was initially

TABLE 2. Differentially expressed sigma S core set genes

Gene	Gene product ^a	Expression ^b at:							
		T1	T2	T3	T4	T5	T6	T7	T8
<i>bolA</i>	BolA transcriptional regulator, stress regulation	0.21	0.02	−0.65	−0.01	−0.03	−0.08	−0.05	−0.61
<i>ygaM</i>	Predicted protein	−0.10	0.08	−0.55	0.30	0.29	−0.31	0.16	0.12
<i>yhiW</i>	GadW transcriptional repressor	−0.09	−0.03	−0.51	−0.04	0.07	−0.24	−0.05	−0.34
<i>yhiX</i>	GadX transcriptional activator	0.08	−0.15	−0.33	−0.08	−0.12	−0.25	−0.18	−0.09
<i>ychK</i>	Hypothetical protein	0.21	−0.02	−1.47	0.15	0.06	−0.01	0.18	−0.36
<i>ugpC</i>	Glycerol-3-P ABC transporter, SU	−0.28	0.00	−0.56	0.10	−0.14	0.09	−0.05	−0.24
<i>yhhA</i>	Conserved protein	−0.01	−0.26	−0.16	−0.45	−0.36	−0.08	−0.28	−0.15
<i>treA</i>	Trehalase, periplasmic	−0.12	−0.02	−0.36	−0.01	0.02	0.03	0.07	−0.01
<i>yhiO</i>	Ethanol tolerance protein	0.05	0.15	0.62	−0.13	0.01	0.34	0.08	0.35
<i>yedU</i>	Hsp31 molecular chaperone, SU	0.10	0.49	0.27	0.51	0.06	−0.17	0.27	0.16
<i>yceK</i>	Predicted lipoprotein	0.20	0.14	0.42	0.02	0.18	−0.01	0.13	0.12
<i>b1758</i>	Predicted phosphatidyl transferase	−0.13	0.07	0.30	−0.14	−0.20	0.22	0.03	0.31
<i>b2086</i>	Conserved protein	−0.29	0.02	0.51	0.01	0.25	0.40	0.28	0.31
<i>hdhA</i>	7-Alpha-hydroxysteroid dehydrogenase, SU	0.11	0.36	0.49	0.11	0.01	−0.10	0.05	0.41
<i>yjeB</i>	NsrR transcriptional repressor	0.10	0.34	0.06	0.11	−0.04	−0.19	0.03	0.11
<i>otsB</i>	Trehalose-6-phosphate phosphatase	0.00	0.30	0.06	−0.04	0.23	0.15	0.32	0.22
<i>rpsV</i>	30S ribosomal subunit protein S22	0.28	0.59	0.51	0.66	1.15	1.13	1.38	1.59
<i>b0753</i>	Putative homeobox protein	0.14	0.57	0.37	0.59	0.34	0.47	0.71	0.61
<i>ygaF</i>	Predicted enzyme	0.24	0.39	0.42	0.44	0.36	0.11	0.27	0.25
<i>yeaG</i>	Conserved protein	0.20	0.08	−0.03	0.47	0.24	0.02	0.04	0.04
<i>ygaE</i>	CsiR transcriptional repressor	0.13	0.27	0.10	0.07	0.11	0.47	0.02	0.14
<i>yjgR</i>	Putative enzyme with P-loop containing nucleotide triphosphate hydrolase domain	0.04	0.03	−0.24	0.27	0.26	0.67	−0.11	−0.04
<i>ygaU</i>	Predicted protein	0.07	0.08	0.26	0.12	0.14	0.30	0.24	0.37
<i>ymgA</i>	Hypothetical protein	−0.19	0.18	0.37	0.12	0.30	0.17	0.29	0.64
<i>b2097</i>	Fructose bisphosphate aldolase class I, SU	−0.03	0.13	0.39	0.39	0.44	−0.05	−0.06	0.36
<i>yjgB</i>	Predicted alcohol dehydrogenase	0.01	0.08	0.64	0.27	0.16	0.14	−0.23	0.14

^a From reference 67.^b Coefficients of the linear model. Underlining indicates significantly differential expression.

believed to be directly involved in chemotaxis (7), its role in chemotaxis was later refuted or considered to be barely “indirect” (66). Chemotaxis and the glucose-specific PTS, however, share some common structural elements, such as the proteins EI, EII, and HPr. Since these proteins are part of the cAMP synthesis pathway, it might be speculated that these elements are cross-linked through the alarmone cAMP. The transcriptome data presented here support previous findings published by Soutourina et al. (62), who put forward the idea of the multiple control of flagellar biosynthesis. In this context, the transcription control of the early class master operon, *flhDC*, through the global regulator protein H-NS and the Crp-cAMP complex is of particular importance. At T2, H-NS expression was increased, while fewer *flhD* transcripts were found (Table 1).

A further issue to be addressed in the discussion about downregulation of the chemotaxis is the proton gradient. Chemotaxis depends on a steep proton gradient between the periplasmic space and the cytoplasm (for a review, see reference 6). Transport phenomena, however, also depend on proton gradients, among others. The experimental observation of the opposite direction in gene regulation of chemotaxis and transport systems could be interpreted as the result of a competition, in which the more effective transport of extant sugar or further energy-supplying compounds seems to be more important than the chemotaxis.

(iv) Cell growth. The specific growth rate decreased strongly during fed-batch cultivation (Fig. 1). A total of 128 genes coding for anabolic enzymes and the salvage pathway were differentially expressed at at least one point in time (see the supplemental material). The investigation suggests that the cells carefully utilized the available external and internal resources for their growth. It can therefore be assumed that the capacity of synthesizing recombinant proteins and metabolites decreases under constant-feeding conditions. Exponential feeding might have a positive effect, although this strategy is also based on the limitation of the carbon source.

(a) Monomer synthesis. Glucose limitation led to the reduced synthesis of mRNAs of genes required for the biosynthesis enzymes of various monomers (amino acids, fatty acids, carbohydrates, and nucleotides) as well as of coenzymes and prosthetic groups (see the supplemental material) (Fig. 2). Although much is known about the regulation of the respective genes through the stringent response, further clarification of the mechanisms and their directionality (i.e., positive or negative) is still necessary (11).

(b) Polymer synthesis (cell composition and cell mass). Most of the genes involved in protein biosynthesis (ribosomal proteins, RNA polymerase subunits, ribosomal assembly proteins, protein maturation proteins, RNA modification proteins, and aminoacyl-tRNA synthetases) were transcribed to a lesser extent (see the supplemental material) (Fig. 4). A total of 78% of

TABLE 3. Differentially expressed stress-related genes

Gene	Gene product	Expression ^a at:							
		T1	T2	T3	T4	T5	T6	T7	T8
<i>ppiA</i>	Peptidyl-prolyl <i>cis-trans</i> isomerase A, chaperoning, repair	-0.25	0.01	0.07	0.11	-0.28	<u>-0.56</u>	0.15	-0.04
<i>degS</i>	Inner membrane serine protease (sigmaE response)	<u>-0.73</u>	-0.07	-0.12	0.06	-0.07	-0.3	-0.17	-0.09
<i>narJ</i>	Chaperone subunit (δ subunit) of nitrate reductase 1	0.03	-0.02	<u>-0.77</u>	-0.04	-0.17	0.21	0.13	0.03
<i>hslJ</i>	Heat shock protein	0.07	-0.15	<u>-0.58</u>	-0.09	0.15	-0.27	0.01	-0.22
<i>hdeA</i>	Acid resistance protein, possible chaperone	<u>0.55</u>	0.11	<u>-0.38</u>	-0.25	-0.34	-0.3	-0.35	-0.37
<i>yabH</i>	Chaperone with DnaK	0.07	-0.2	-0.07	-0.21	<u>-0.57</u>	-0.04	0.13	<u>-0.53</u>
<i>stpA</i>	H-NS-like DNA-binding protein with RNA chaperone activity	-0.04	<u>-0.58</u>	<u>-0.4</u>	-0.31	-0.29	-0.31	0.01	<u>-0.72</u>
<i>bcp</i>	Thiol peroxidase (detoxification)	0.19	-0.17	<u>-0.53</u>	-0.23	-0.42	-0.32	-0.04	<u>-0.55</u>
<i>ydaA</i>	Universal stress protein (resistance to UV irradiation)	0	-0.16	<u>-0.39</u>	<u>-0.37</u>	-0.22	-0.33	-0.14	-0.14
<i>recF</i>	Subunit of RecFOR complex, DNA recombination, replication, repair	0.09	-0.27	-0.17	<u>-0.45</u>	-0.14	-0.31	-0.27	-0.17
<i>hslV</i>	Peptidase component of the HslVU protease, chaperoning, repair	0.08	-0.13	-0.19	<u>-0.4</u>	-0.2	-0.28	-0.33	-0.29
<i>dnaJ</i>	Chaperone, heat shock protein	0.05	-0.39	-0.31	-0.05	-0.11	-0.12	<u>-0.63</u>	0.1
<i>ymdD</i>	Protein required for succinyl modification of osmoregulated periplasmic glucans	0.03	0.27	0.11	0.04	-0.16	0.14	-0.13	<u>0.55</u>
<i>mutL</i>	Methyl-directed mismatch repair, SU	0.02	-0.02	0.18	0.22	0.03	-0.41	0.43	<u>1.04</u>
<i>yhiO</i>	Ethanol tolerance protein	0.05	0.15	<u>0.62</u>	-0.13	0.01	0.34	0.08	0.35
<i>msrA</i>	Protein-methionine-S-oxide reductase, chaperoning, repair	0.13	0.23	<u>0.41</u>	0.09	0	0.11	0.05	0.13
<i>sodA</i>	Superoxide dismutase, SU	-0.05	-0.07	<u>0.24</u>	0	0.04	0.17	0.01	0.07
<i>ydeB</i>	Inner membrane protein involved in multiple antibiotic resistance	0	-0.03	<u>0.49</u>	-0.1	-0.04	-0.04	0.02	0.13
<i>uvrB</i>	UvrABC nucleotide excision repair complex, SU	0.19	-0.07	<u>0.94</u>	0.07	-0.11	-0.06	-0.01	-0.24
<i>yedU</i>	Hsp31 molecular chaperone, SU	0.1	0.49	0.27	<u>0.51</u>	0.06	-0.17	0.27	0.16
<i>ydgO</i>	Integral membrane protein of SoxR-reducing complex	0.03	<u>0.28</u>	<u>0.25</u>	0.03	0.04	-0.04	0.02	0.01
<i>cutC</i>	Copper homeostasis protein, detoxification	0.18	0.08	0.33	0.44	0.5	0.25	0.55	<u>0.74</u>
<i>ybeV</i>	Hsc56, cochaperone of Hsc62	0	<u>0.43</u>	<u>0.55</u>	0.17	0.26	<u>0.43</u>	0.16	0.28
<i>cspI</i>	Qin prophage, cold shock protein	-0.06	<u>0.47</u>	0.36	0.36	0.15	0.47	0.33	0.33
<i>yeeA</i>	Protein-methionine-S-oxide reductase, chaperoning, repair	0.29	<u>0.45</u>	<u>0.69</u>	<u>0.64</u>	0.47	0.44	<u>0.66</u>	<u>0.93</u>
<i>ahpC</i>	Alkylhydroperoxide reductase, SU, detoxification	0.17	<u>0.44</u>	<u>0.6</u>	<u>0.61</u>	0.33	0.27	0.32	<u>0.49</u>
<i>phoH</i>	ATP-binding protein, induced by P starvation	0.09	-0.02	0.06	-0.07	-0.2	<u>0.53</u>	-0.01	0.16
<i>cspF</i>	Qin prophage, cold shock protein	-0.01	-0.02	0.12	0.04	-0.07	<u>0.69</u>	-0.04	0.06
<i>b1631</i>	Member of SoxR-reducing complex	0.04	0.06	0.04	-0.07	0.05	<u>0.36</u>	0.04	-0.03
<i>b0245</i>	Toxin of the YkfI-YafW toxin-antitoxin pair	-0.08	0.16	0.22	-0.21	-0.08	<u>0.51</u>	0.07	0.04
<i>sbmC</i>	DNA gyrase inhibitor	0.02	-0.01	0.09	0.22	<u>0.43</u>	0.16	0.03	-0.15
<i>mutH</i>	MutHLS complex, SU, methyl-directed mismatch repair	-0.01	0.06	0.03	<u>0.48</u>	0.11	-0.28	0.13	-0.23
<i>rpoE</i>	Sigma E factor	0.22	0.09	0.23	<u>0.65</u>	<u>0.79</u>	0.52	<u>0.95</u>	<u>0.83</u>

^a Coefficients of the linear model. Underlining indicates significantly differential expression.

the genes encoding ribosomal proteins were transcribed to a lesser extent. The gene for the 30S ribosomal subunit protein S22 (encoded by *rpsV*) is expressed at higher levels during the stationary phase (32) and was transcribed more actively during the fed-batch period. Most of the underlying regulatory mechanisms are well known and can be assigned to the stringent response (11). It can be safely assumed that these mechanisms lead to a change in the macromolecular composition of the cell.

(c) *Cell division*. Sixteen genes coding for cell division enzymes (see the supplemental material) (Fig. 2) whose transcript levels decreased (e.g., *ftsAZ*, *tig*, and *seqA*) were identified; the number of transcripts of cell division inhibition enzymes (*minCD* and *cspD*) increased.

(d) *Macromolecular degradation*. Fifty-one genes coding for enzymes that degrade carbohydrates, amino acids, fatty acids, nucleic acids, and nucleotides were differentially expressed at at least one time point under glucose-limiting conditions (see the supplemental material) (Fig. 2).

(v) *Stress and starvation response*. According to Ferenci (18), the nutritional state of bacteria can be separated into "feast" (glucose rich) and "famine" (glucose starved). The physiological response of *E. coli* to glucose limitation provides evidence for the further separation between "hunger" and "starvation" responses (20). Ferenci assumed that the rapid

sequence of hunger and starvation responses occurred when the batch cultures were grown until depletion of a nutrient during conventional starvation experiments. The fed-batch procedure chosen in the present study enabled the investigation of the dynamic decline of the growth rate due to the decreasing glucose levels. This might provide further insights in support of Ferenci's hypothesis. This is not only of pure academic interest, because the majority of production processes (e.g., high-cell-density cultivations for recombinant protein, amino acid, or antibiotic production) are performed under these stress conditions and may hence suffer from stress-related protein turnover.

It is generally assumed that the starvation response occurs during the stationary phase, which is characterized by a complete exhaustion of nutrients (C, N, P, or S) and which is mediated by the stationary-phase sigma factor (σ^S , encoded by *rpoS*) (29). Weber et al. (67) carried out global transcription analyses and identified 140 genes as a core set of σ^S -regulated genes. In the present fed-batch experiment, only 18 of these genes showed elevated transcript levels at at least one point in time (Table 2). This number corresponds to 2% of all genes identified in the present study. This implies that carbon limitation and the concomitant stringent response do not necessarily lead to the σ^S -mediated stress response during glucose-limited growth (Fig. 2). However, 33 genes associated with

TABLE 4. Differentially expressed sigma S-regulated genes

Gene	Gene product ^a	Expression ^b at:							
		T1	T2	T3	T4	T5	T6	T7	T8
<i>himD</i>	Integration host factor transcriptional dual regulator, SU	0.02	0.20	0.07	<u>0.50</u>	0.22	0.10	-0.01	-0.21
<i>htrE</i>	Putative outer membrane porin protein involved in fimbrial assembly	-0.21	-0.10	<u>0.49</u>	0.09	0.35	<u>0.49</u>	0.14	0.42
<i>proW</i>	Proline ABC transporter UE	-0.07	-0.05	-0.12	0.01	-0.09	-0.11	0.18	<u>0.48</u>
<i>csiE</i>	Stationary-phase-inducible protein	0.07	0.09	0.01	<u>0.36</u>	0.31	0.15	0.15	0.36
<i>rpsV</i>	30S ribosomal subunit protein S22	0.28	<u>0.59</u>	0.51	<u>0.66</u>	<u>1.15</u>	<u>1.13</u>	<u>1.38</u>	<u>1.59</u>
<i>yehX</i>	YehW/YehX/YehY/YehZ ABC transporter subunit	-0.16	0.00	0.25	0.03	-0.01	0.08	-0.05	<u>0.42</u>
<i>yehY</i>	YehW/YehX/YehY/YehZ ABC transporter subunit	0.10	-0.21	0.10	0.20	0.43	<u>0.73</u>	-0.05	0.01
<i>yelL</i>	Transcriptional activator	0.00	0.18	<u>0.42</u>	0.15	0.14	0.23	-0.02	0.27
<i>ygaF</i>	Predicted enzyme	0.24	<u>0.39</u>	<u>0.42</u>	<u>0.44</u>	0.36	0.11	0.27	0.25
<i>yhiO</i>	Ethanol tolerance protein	0.05	0.15	<u>0.62</u>	-0.13	0.01	0.34	0.08	0.35
<i>proV</i>	Proline ABC transporter UE	-0.32	<u>-0.40</u>	-0.08	-0.12	-0.11	-0.11	0.36	<u>1.14</u>
<i>hdeA</i>	Acid resistance protein, possible chaperone	<u>0.55</u>	0.11	<u>-0.38</u>	-0.25	-0.34	-0.30	-0.35	-0.37
<i>treA</i>	Trehalase, periplasmic	-0.12	-0.02	<u>-0.36</u>	-0.01	0.02	0.03	0.07	-0.01
<i>recF</i>	RecFOR complex, SU	0.09	-0.27	-0.17	<u>-0.45</u>	-0.14	-0.31	-0.27	-0.17
<i>bolA</i>	BolA transcriptional regulator, stress regulation	0.21	0.02	<u>-0.65</u>	-0.01	-0.03	-0.08	-0.05	-0.61
<i>yhiX</i>	GadX transcriptional activator	0.08	-0.15	<u>-0.33</u>	-0.08	-0.12	-0.25	-0.18	-0.09
<i>pqiB</i>	Paraquat-inducible protein B	-0.11	-0.36	-0.40	-0.22	-0.27	<u>-0.57</u>	-0.38	-0.42
<i>caiC</i>	Carnitine-coenzyme A ligase/crotonobetaine-coenzyme A ligase	0.07	-0.32	0.04	0.06	-0.33	<u>-0.83</u>	-0.14	0.02
<i>ftsA</i>	Essential cell division protein	0.07	-0.05	-0.09	0.01	<u>-0.45</u>	-0.32	-0.18	0.03
<i>galT</i>	Uridyltransferase, galactose metabolism	0.01	0.13	<u>-0.56</u>	-0.04	0.13	-0.21	0.06	-0.14

^a From the Ecocyc.org database.^b Coefficients of the linear model. Underlining indicates significantly differential expression.

stress conditions (heat and cold shock or oxidative stresses) were differentially expressed (Table 3; Fig. 2). Only three of these genes (*hdeA*, *recF*, and *yhiO*) are regulated by the sigma S factor (Table 4).

The alarmone ppGpp has a positive effect on *rpoS* transcript levels (24), while the Crp-cAMP complex inhibits *rpoS* transcription. In our previous study, the concentrations of these two alarmones were elevated during glucose limitation (27). It has already been suggested that these opposing regulations could result in the reduction of *rpoS* transcription during glucose-limited growth in bioreactors (36). However, other known regulatory mechanisms may also be worth considering (29).

The global regulator LrhA seems to diminish the σ^S level (47). In our studies, an increase of the *lrhA* mRNA level was observed at T3 until the end of the cultivation (Table 1). The effect of LrhA on the σ^S level might be a reason for the lack of a σ^S response. The increased transcription of the global regulator protein H-NS from T3 to T6 might be an explanation for the lack of a σ^S -mediated response. H-NS binds to *rpoS* mRNA and enhances its cleavage (10). However, one needs to keep in mind that all of these effects are balanced and lead to constant *rpoS* transcript levels.

The global transcription analysis presented in this study confirms the findings of Teich et al. (65), who suggested that slow glucose concentration changes significantly increase the cell's ability to adapt to new physiological states without using the rearrangements mediated by the σ^S stress response. Therefore, it may be concluded that the strategy of constant nutrient feeding contributes mainly to the hunger state but is less important in terms of the cell's stress-induced starvation.

Conclusions. The current contribution globally analyzed time-dependent transcript and metabolic flux levels in *E. coli* K-12 W3110 fed-batch cultures. It was possible to simultaneously track the carbon limitation responses (in the transport systems, intermediary metabolism, growth-related functions,

chemotaxis, and stress response), which illustrates the power of the experimental setup used. The constant-feeding strategy also provided an appropriate approach for separating the time-dependent events during the transition from exponential growth to strong carbon limitation. The novelty of this work arises from the integration of the dynamic transcriptional, metabolic, and regulatory responses into a comprehensive hypothetical model (Fig. 2 and 3b and c), pinpointing the impact of these variations on the general employment of the available cellular resources.

Previous findings show that the flux redistribution during carbon-limited growth resulted in a significantly lower biomass yield, which is mainly due to the oxidation of the substrate in the TCA cycle for the generation of energy (27). The current findings led to the hypothesis that the general rate of oxidative decarboxylation can be limited by regulating the EMP/PPP, RpiA/Rpe, and TCA/GS/PEP-GS split ratios. Accordingly, an optimal carbon and energy balance of the central carbon metabolism (homeostasis) will be achieved. Nevertheless, the split ratios need to be investigated in more detail in order to validate the results from the above-mentioned stoichiometric metabolite balancing method. Flux analyses using isotopic transient ¹³C labeling data may be the method of choice, as they have become technically possible for fed-batch processes (40, 44, 55).

The results obtained in this investigation strongly support the hypothetical regulatory model structure put forward by Hardiman et al. (27). Many other regulatory mechanisms might have minor effects on *E. coli* metabolism (27), particularly in case of fed-batch processes with recombinant *E. coli* strains; however, the global genetic regulatory systems discussed are considered most relevant for the control of behavior during carbon-limited growth. This model, which is an extension of the previous dynamic metabolic model of Chassagnole et al. (12), is the basis for ongoing research relating to the

mathematical modeling of the dynamics occurring in the central carbon metabolism of *Escherichia coli* K-12 W3110. The work provides a step forward toward the more detailed understanding of the impacts of carbon limitation on metabolic activities that must be taken into account when optimizing biotechnological processes. For example, the regulation of flux splitting might be an appropriate target for counteracting the excessive loss of carbon and energy in carbon-limited processes via oxidative decarboxylation and thus for the optimization of the yields of biomass, recombinant proteins, and other products.

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