Global gene expression in *Escherichia coli* K-12 during short-term and long-term adaptation to glucose-limited continuous culture conditions

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Microarray technology was used to study the cellular events that take place at the transcription level during short-term (physiological) and long-term (genetic) adaptation of the faecal indicator bacterium *Escherichia coli* K-12 to slow growth under limited nutrient supply. Short-term and long-term adaptation were assessed by comparing the mRNA levels isolated after 40 or 500 h of glucose-limited continuous culture at a dilution rate of 0.3 h^{-1} with those from batch culture with glucose excess. A large number of genes encoding periplasmic binding proteins were upregulated, indicating that the cells are prepared for high-affinity uptake of all types of carbon sources during glucose-limited growth in continuous culture. All the genes belonging to the maltose (*mal/lamB*) and galactose (*mgll/gal*) operons were upregulated. A similar transcription pattern was observed for long-term cultures except that the expression factors were lower than in the short-term adaptation. The patterns of upregulation were confirmed by real-time RT-PCR. A switch from a fully operational citric acid cycle to the PEP-glyoxylate cycle was clearly observed in cells grown in glucose-limited continuous culture when compared to batch-grown cells and this was confirmed by transcriptome analysis. This transcriptome analysis confirms and extends the observations from previous proteome and catabolome studies in the authors' laboratory.

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INTRODUCTION

Worldwide, *Escherichia coli* is used as a bacterium to indicate faecal contamination of (drinking) water (WHO, 1996) and *E. coli* has been extensively investigated in the laboratory with respect to its physiology, biochemistry and genetics. Despite this, surprisingly little is known about how this bacterium grows, competes and survives in its primary (i.e. warm-blooded hosts) (Conway, 1995; Macfarlane & Macfarlane, 1997, 2004) and secondary habitats (i.e. soil and water) (Rozen & Belkin, 2001; Savageau, 1983). In contrast to nutrient-rich laboratory cultures, growth conditions in the aqueous environment, such as surface, ground and drinking water, are much harsher, and concentrations of utilizable carbon sources are typically in the low $\mu\text{g l}^{-1}$ range (Egli, 1995; Morita, 1993; Münster, 1993). Similarly, access to nutrients in soil is restricted most of the time (Williams, 1985). Hence, growth of heterotrophic microbes in natural environments is carbon/energy-limited most of the time.

It has long been the dogma that cells of *E. coli* cannot survive in secondary habitats and that they die off quickly. However, much controversial information can be found in the literature on survival and growth of this bacterium in different natural environments (Hazen & Toranzos, 1990). Recent developments in detection techniques indicate longer survival in the environment than observed with the traditional plate count method (Garcia-Armisen & Servais, 2004). For example, growth of *E. coli* was observed particularly in warmer and polluted fresh water (McFeters, 1990), and Rozen and coworkers have reported that *E. coli* does not die off quickly, but survives long periods of starvation and can even grow in sea water (Rozen & Belkin, 2001).

There is not much conclusive information available on the physiological state of *E. coli* in its primary and secondary habitats (Rozen & Belkin, 2001; Savageau, 1983). One of the experimental approaches to understanding the behaviour of this bacterium under carefully controlled nutritional conditions is the use of continuous culture (Egli, 1995; Macfarlane *et al.*, 1998). In particular carbon/energy-limited continuous culture has been used to study the kinetic and physiological properties of micro-organisms during slow growth. Interestingly, during the cultivation of *E. coli* in glucose-limited continuous culture two phases of adaptation to such growth conditions were detected and they

Abbreviations: PEP, phosphoenolpyruvate; TCA, tricarboxylic acid.

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Four supplementary tables with additional gene expression data are available with the online version of this paper.

can be identified based on kinetic and physiological properties of the cells (Wick *et al.*, 2001). A first phase, the so-called short-term adaptation phase, follows immediately after the culture has reached the steady state with respect to biomass and residual glucose concentration. This phase lasts no longer than approximately 50–70 generations. Changes in the proteome/cellular composition during this phase occur solely at the physiological level by regulation of gene expression at the transcriptional and translational level. Generally, an increased expression of alternative carbon/energy and nitrogen source transport systems and increased expression of components of stress response regulons are observed (Hua *et al.*, 2004; Ihssen & Egli, 2004; Wick *et al.*, 2001). In strain K12 this phase is characterized by a constant affinity for glucose, with a K_s of $\sim 400 \mu\text{g l}^{-1}$, which is a slight improvement compared to growth at glucose-excess concentrations in batch culture ($K_s \sim 600 \mu\text{g l}^{-1}$). This short-term adaptation phase is followed by a mutant selection phase (50–500 generation times in the chemostat) in which mutants with increased affinity for the growth-limiting glucose are enriched (Wick *et al.*, 2002). This so-called long-term adaptation leads to a 10–15-fold increase in the affinity for glucose. It was shown that this phase is characterized by the selection of regulatory mutants affected in the expression of the *mgl/mal* system (Notley-McRobb & Ferenci, 1999a, b). These changes have been well documented at the proteome (Wick *et al.*, 2001) and catabolome (Ihssen & Egli, 2005) levels, as well as the genetic level (Notley-McRobb & Ferenci, 1999a, b).

Therefore, the analysis of the transcriptome is an additional tool for an improved understanding of the bacterial response to carbon/energy limitation. The advent of microarray technology has increased the interest in the study of the global gene expression under different environmental conditions. The understanding of global gene expression profiles under different nutritional conditions is very important for the understanding of the growth of bacteria in their natural environment.

The purpose of the work presented here was to identify the genes that are differentially expressed during the adaptation to low glucose concentration in glucose-limited continuous culture as compared to growth with excess glucose during cultivation in batch culture. Special attention was given to the uptake systems and the regulatory proteins involved in the evolution occurring during adaptation to low glucose concentrations. The results obtained in this study are compared with the results obtained from the proteome (Wick *et al.*, 2001) and the catabolome (Ihssen & Egli, 2005), where the same process was documented by different analytical methods. One of the major advantages of the microarray technology is that all genes can be analysed and compared, whereas with the other two methods only a limited set of proteins of physiological information can be obtained. However, for a complete rounded picture one needs all the different methods that give information at complementary levels.

METHODS

Bacterial strain and growth. Wild-type *Escherichia coli* K-12 MG1655 (ATCC 700926) was grown at 37°C in mineral medium allowing carbon-limited cultivation up to a glucose concentration of 2 g l^{-1} . The mineral medium consisted of $1.0 \text{ g KH}_2\text{PO}_4$, $1.15 \text{ g NH}_4\text{Cl}$, $0.1 \text{ ml concentrated H}_2\text{SO}_4$ and $0.02 \text{ ml silicone anti-foam}$ (Fluka) per litre. The mineral medium for preculture, allowing the supply of glucose concentrations up to 6 g l^{-1} , consisted of $12.8 \text{ g Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, $3.0 \text{ g KH}_2\text{PO}_4$ and $1.77 \text{ g (NH}_4)_2\text{SO}_4$ per litre. Glucose stock solution, salt and trace elements (100-fold concentrated solution) were added after autoclaving and cooling. The trace elements were present at final concentrations of: $26.8 \text{ mg MgCl}_2 \cdot 6\text{H}_2\text{O l}^{-1}$; $16 \text{ mg CaCO}_3 \text{ l}^{-1}$; $15.5 \text{ mg FeCl}_3 \cdot 6\text{H}_2\text{O l}^{-1}$; $2.3 \text{ mg MnCl}_2 \cdot 4\text{H}_2\text{O l}^{-1}$; $0.3 \text{ mg CuSO}_4 \cdot 5\text{H}_2\text{O l}^{-1}$; $0.3 \text{ mg CoCl}_2 \cdot 6\text{H}_2\text{O l}^{-1}$; $0.8 \text{ mg ZnO l}^{-1}$; $0.25 \text{ mg H}_3\text{BO}_3 \text{ l}^{-1}$; $2.1 \text{ mg NaMoO}_4 \cdot 2\text{H}_2\text{O l}^{-1}$ and $158.4 \text{ mg Na}_4\text{EDTA} \cdot 2\text{H}_2\text{O l}^{-1}$ (equimolar to di- and trivalent cations).

Growth was monitored by three different methods. Cell number was measured by plating on tryptic soy agar (TSA; Biolife) plates after appropriate dilution and, for assessing purity, on ECD-MUG agar plates (Biolife). Dry cell weight was measured by filtration onto $0.22 \mu\text{m}$ membrane filters (Millipore) and drying to constant weight at 105°C . Furthermore, culture density was monitored spectrophotometrically by measuring optical density at 546 nm (OD_{546}) in a 1 cm cuvette (Uvikon 930; Kontron).

Cultivation conditions. For batch cultures of *E. coli* mineral medium with $1 \text{ g glucose l}^{-1}$ was used. For glucose-limited continuous cultures the concentration of glucose in the feed medium was reduced to 0.1 g l^{-1} in order to be able to follow the kinetics of adaptation at the level of residual glucose concentrations (Senn *et al.*, 1994). Cultivation in both batch and continuous culture was performed in a computer-controlled glass and stainless steel bioreactor with a 2 litre working volume (MBR). The stirrer speed was set to 800 r.p.m. , oxygen concentration was kept over 90% of air saturation, the temperature was controlled at $37 \pm 0.1^\circ\text{C}$ and pH was maintained at 7.0 ± 0.1 by automatic addition of a mixture of NaOH and KOH (0.5 M each).

For each growth experiment, cultures were always started freshly from the same cryo-vial stored at -80°C to avoid as much as possible the picking up of mutants due to adaptation to the growth medium. Cells were streaked out first on TSA plates and after 24 h a colony was transferred into 5 ml mineral medium with $4 \text{ g glucose l}^{-1}$. When this first pre-culture had reached an OD_{546} of $0.3\text{--}0.4$, 1 ml of the culture was transferred to 100 ml of the same medium. Cultures in bioreactors were inoculated with 60 ml of this second pre-culture when it reached an OD_{546} of 0.3 . Growth was followed by measuring OD_{546} at regular intervals to confirm that cells were growing at μ_{max} ($0.63 \pm 0.02 \text{ h}^{-1}$). A sample for transcriptome analysis was taken from the batch exponential phase at an OD_{546} of ~ 0.4 , and immediately after sampling the medium flow was started for continuous cultivation with a dilution rate of 0.3 h^{-1} . Steady state with respect to biomass and residual glucose concentrations was reached approximately 15 h after this manipulation. At 40 and 500 h after switching to the glucose-limited continuous culture mode, 800 ml of culture liquid was withdrawn from the chemostat and immediately cooled on crushed ice, and cells were collected by centrifugation at 4°C . From the resulting cell pellet RNA was instantaneously isolated (see below). Residual glucose concentrations were monitored during the continuous cultures run; samples were filtered as described earlier (Senn *et al.*, 1994) and analysed by ion chromatography (Ihssen & Egli, 2004). The K_s and the sequence of mutant populations during the adaptation process were simulated using AQUASIM according to Wick *et al.* (2002).

RNA isolation and synthesis of cDNA. Total RNA from *E. coli* cells was isolated as described by Sambrook *et al.* (1989). RNA in samples was quantified spectrophotometrically by measuring absorbance at 260 nm, and purity (freedom from DNA) was checked by gel electrophoresis. Synthesis of cDNA from RNA was performed with the CyScribe First-Strand cDNA Labelling Kit (Amersham Bioscience). Reverse transcription was performed using 25 µg total RNA (maximally in 10 µl) and 1 µl random nonamer primers. The volume of the assay mixture was adjusted to 11 µl with RNase-free water, then the assay mixture was incubated for 5 min at 70 °C, followed by incubation for 10 min at room temperature to allow the primers to anneal with the RNA. After cooling to room temperature, the reagents for the labelling reaction were added. After the addition of 4 µl 5 × CyScript buffer, 2 µl 0.1 M DTT, 1 µl dNTP mixture, 1 µl 0.5 mM either Cy3-labelled or Cy5-labelled dCTP (Amersham Bioscience) and 1 µl CyScript reverse transcriptase (100 U µl⁻¹), the final reaction volume was 20 µl. The control cDNA (from batch-grown cells) and the probe cDNA (from either the 40 h old or the 500 h old chemostat culture) were labelled differently, with the control cDNA always labelled with Cy5. The labelling reaction was performed at 42 °C for 1.5 h, followed by RNA degradation and cDNA purification. The RNA was degraded by addition of 2 µl 2.5 M NaOH; the mixture was then heated at 65 °C for 10 min and subsequently neutralized with 10 µl 2 M HEPES buffer. The control and probe cDNA obtained were pooled and purified on the same column of the MinElute Gel Extraction Kit (Qiagen) to avoid differences in extraction yields.

DNA microarray, hybridization and washing. Slide microarrays were purchased from MWG-Biotech. The MWG *E. coli* array contains 4288 gene-specific oligonucleotide probes representing the complete *E. coli* K-12 genome. The purified cDNA was concentrated to 5 µl and was mixed with 120 µl hybridization buffer (MWG-Biotech), heated to 95 °C for 3 min and cooled on ice for 3 min. The hybridization mixture was then added to the microarray slide and covered with a coverslip. The hybridization slide was incubated overnight at 42 °C. After the hybridization step the slide was washed three times, the first time for 5 min in 2 × SSC/0.1% SDS, the second time for 5 min in 1 × SSC, and finally for 5 min in 0.1 × SSC. SSC buffer was prepared as a 20 × solution containing 0.3 M sodium citrate and 3 M NaCl at pH 7.0. The slides were dried by centrifugation at room temperature for 2 min at 500 g.

Image and data analysis. Microarray slides were scanned using an Affymetrix 428 Array Scanner. Spot intensities and corresponding background signals were quantified with the Affymetrix Jaguar software version 2.0. Further data analysis was performed with the program GeneSpring from Silicon Genetics. Induction factors were calculated from the Cy3 and Cy5 signal intensities of the spot. Spots with signal intensity below a value of 50 were excluded from the analysis and the minimal induction factor was set to 0.01. The normalization was performed with the 50th percentile distribution of remaining spots after background correction. The mean value of the induction factors of a specific gene was calculated from three replicates. Biological experiments were carried out three times, which provided three biological repeats. Data from the independent experiments were combined; genes that were differentially regulated ≥ 2 and ≤ 0.5 (*t*-test, $P \leq 0.05$) were defined as being statistically significant.

Real-time RT-PCR. cDNA was synthesized with SuperScript reverse transcriptase (Invitrogen). The mixture for RNA transcription was prepared for 40 reactions, mixing 0.8 µg total RNA (DNase-digested) with 2 µl random hexamer primers (Invitrogen). The total volume was adjusted to 9 µl with RNase-free water, followed by incubation for 5 min at 65 °C to melt secondary structures, and then cooling for 2 min on ice to anneal the primers. Subsequently, reverse transcriptase reaction components were added, consisting of 0.5 µl RNase inhibitor (Sigma), 4 µl dNTP mixture (2.5 mM each), 2 µl 0.1 M

DTT, 4 µl 5 × reaction buffer and 0.5 µl SuperScript reverse transcriptase. The reaction mixture was mixed and spun down briefly and was then incubated for 10 min at 25 °C for annealing (90 min at 39 °C) reverse-transcribed mRNA. The reaction was stopped by incubation at 95 °C for 5 min. The total volume was adjusted to 40 µl before starting real-time PCR. Real-time PCR was conducted according to the manufacturer's instructions; the assay contained 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 9.5 µl RNase-free water, 1 µl forward primer, 1 µl reverse primer and 1 µl cDNA (20 ng), or the same amount of RNA as negative control. Primers (Microsynth) were designed with the software Primer Express v2.0 (Applied Biosystems) for 12 genes (Table 1), and their optimization (to avoid concatemers or similar problems) was performed by mixing different concentrations of forward and reverse primers. The optimized primer concentration was used to analyse RNA samples. The real-time PCR reaction was conducted using ABI Prism 7000 (Applied Biosystems). The results were normalized using the gene *rrsB* as endogenous control. Real-time PCR data were analysed with ABI Prism 7000 SDS software version 1.0 according to the System User Bulletin #2 'Relative Quantification of Gene Expression' (P/N 4303859) from Applied Biosystems.

RESULTS

Overview of transcriptome analysis of cells cultivated under different growth conditions

The mRNA isolated from cells cultivated in continuous culture under glucose-limited conditions ($D=0.3 \text{ h}^{-1}$) was

Table 1. Primers used in the real-time PCR experiments

Gene	Primer	Sequence (5'–3')
<i>rrsB</i>	K1 fw	GAATGCCACGGTGAATACGTT
	K1 rev	ACCCACTCCCATGGTGTGA
<i>katE</i>	K2 fw	TCCCTTGCGGCAATATCG
	K2 rev	AAGGTGTTTGTAGGCTTCCATCAG
<i>mgIA</i>	rtmgIA fw	AACGTTGCACGGCAAACA
	rtmgIA rev	TACCAAGTCAAATCCATGGTTTA
<i>mgIB</i>	rtmgIB fw	CCTGGCCGATGGTAAAGGT
	rtmgIB rev	AGGTACGCGGACCACTTTGT
<i>mgIC</i>	rtmgIC fw	GTATCGGCTCTGCCACCAA
	rtmgIC rev	ACGCACGCCGCGATAG
<i>lamB</i>	rtlamB fw	GAAACCGCGAACGACGTT
	rtlamB rev	CCGCCCGGGTTGATTT
<i>malE</i>	rtmalE fw	GCCGCCACCATGGAAG
	rtmalE rev	CGGACATCTGCGGGATGT
<i>dppA</i>	rtddpA fw	TGATGTGGTGTCTCGTTCGAT
	rtddpA rev	TGCCGCCAGAAACCTTATGG
<i>rbsB</i>	rttrbsB fw	GGCGAACGTGCAGGACTTA
	rttrbsB rev	GGAGTCGGTCGGGTAAATCA
<i>ugpB</i>	rtugpB fw	CCGCGTGATTGTGGATGA
	rtugpB rev	TGCTGTCTGTGGTGTCTTCT
<i>aldA</i>	rtaldA fw	ATGCAGGCGGTTCAATTTG
	rtaldA rev	CGGCCCCATCGCAAT
<i>rpoS</i>	rttrpoS fw	CCGAGACTGTGGCACAA
	rttrpoS rev	GGGAAGACGCGGATGCTT

Fig. 1. Time-course of the residual glucose concentration in glucose-limited continuous culture of *E. coli* MG1655 operated at $D=0.3\text{ h}^{-1}$. Arrows indicate the time points where mRNA samples were taken. The line indicates the best fit to experimental data of the evolution of the residual glucose concentration due to the succession of mutants with improved affinity modelled with AQUASIM according to Wick *et al.* (2002).

compared with mRNA isolated from batch cultures (exponential phase, $\mu_{\max} 0.63\text{ h}^{-1}$, at $\text{OD}_{546} 0.4$). Two different time-points were chosen for collecting cells and extracting the mRNA (from the chemostat): the first time-point was from the physiological (short-term) adaptation phase and the second one at the end of the genetic (long-term) adaptation phase (Fig. 1). The affinity of the culture for glucose was monitored throughout the continuous culture process and at the two time-points it differed by a factor of more than 10 (K_s from $463 \pm 46\text{ }\mu\text{g l}^{-1}$ to $42 \pm 13\text{ }\mu\text{g l}^{-1}$) (Fig. 1), demonstrating the adaptability of *E. coli* to low glucose concentrations.

The physiological (short-term) adaptation during carbon-limited growth is characterized primarily by an over-expression of high-affinity transporter genes, and the genetic (long-term) adaptation consists of the generation and selection of mutants with an improved affinity for glucose (Wick *et al.*, 2002). In *E. coli* growing under glucose-excess conditions, this sugar enters the cell via the porins OmpC/OmpF (outer membrane) and the transport protein PtsG (inner membrane). However, a high-affinity pathway is induced during limited availability of glucose. Interestingly, it is not the PtsG, but transporters from the maltose (*mal/lamB*) and galactose (*mgl/gal*) operons which seem to be involved in high-affinity uptake of glucose (Ferenci, 1996). A strongly increased expression of *mglB* and *malE* (galactose- and maltose-binding proteins, respectively) in chemostat-grown cells (Wick *et al.*, 2001) confirmed the importance of the *mgl* and *mal* gene products for high-affinity glucose uptake. Among the upregulated genes monitored in our study, the transcript levels of the maltose and galactose high-affinity uptake systems were upregulated significantly (Table 2), which is in agreement with earlier proteome data (Wick *et al.*, 2001). Surprisingly, *ptsH* and *crr* (not included in Table 4 because their expression factors were > -10 , see supplementary data), two genes of the glucose PTS, were slightly down-regulated. This result could be a consequence of the fact that the main flux of glucose occurs via the maltose and galactose high-affinity systems under the conditions tested (Death & Ferenci, 1993b; Death *et al.*, 1993a; Ferenci, 1996).

In addition to the higher glucose affinity, the evolved population has a 10 % higher maximum specific growth rate ($\mu_{\max}$) (Wick *et al.*, 2001), due to (an unknown) genetic adaptation. Multiple *mal*- and *mgl*-regulatory mutations are selected for and accumulate during growth in glucose-limited chemostats. The selected population is heterogeneous with respect to the location of the mutation but all of the mutations result in an increased affinity for glucose

Table 2. Relative expression levels of *E. coli* genes belonging to the maltose and galactose high-affinity transport systems after 40 and 500 h in glucose-limited continuous culture ($D=0.3\text{ h}^{-1}$)

Gene	b no.	Gene product	Ratio	
			40 h	500 h
<i>mglC</i>	b2148	Methyl-galactoside transport	7.75	2.37
<i>mglA</i>	b2149	ATP-binding component of methyl-galactoside transport	13.56	3.84
<i>mglB</i>	b2150	Periplasmic galactose-binding protein	27.99	21.79
<i>malT</i>	b3418	Positive regulator of <i>mal</i> regulon	3.59	1.26
<i>malG</i>	b4032	Part of maltose permease, inner membrane	30.51	14.25
<i>malF</i>	b4033	Part of maltose permease, periplasmic	55.19	6.68
<i>malE</i>	b4034	Periplasmic maltose-binding protein	67.39	15.79
<i>malK</i>	b4035	ATP-binding component of transport system for maltose	15.77	4.75
<i>lamB</i>	b4036	Phage lambda receptor protein, maltose high-affinity receptor	32.14	9.15
<i>malM</i>	b4037	Periplasmic protein of <i>mal</i> regulon	9.40	4.51

uptake (Notley-McRobb & Ferenci, 1999a, b). However, the major mutations were found in genes of the maltose and galactose operons (Notley-McRobb & Ferenci, 1999a, b), and in *rpoS* (Notley-McRobb *et al.*, 2002). The maltose and galactose systems were overexpressed after 500 h in a glucose-limited chemostat when compared with cells collected from the physiological adaptation phase. At the mRNA level, the maltose and galactose high-affinity uptake systems were also upregulated, but their expression factor was less pronounced than after 40 h of continuous culture (Table 2).

Changes in mRNA levels during the physiological (short-term) adaptation

As a result of the physiological adaptation, which was examined here after 40 h of glucose-limited continuous culture, 340 genes (see Supplementary Table S1, available with the online version of this paper) were upregulated by a factor of ≥ 2 in comparison to batch culture (Fig. 2a). On the other hand, 502 genes were detected with down-regulated expression levels by a factor of ≤ -2 (Supplementary Table S3), of which more than 37% (187 genes) were of miscellaneous (few) or hypothetical (most) function (Fig. 2b). Among the upregulated genes, the transcript levels of 141 genes were significantly elevated above a factor of 5 (Table 3). In this group of genes the maltose and galactose operons were omitted, because these are summarized in Table 2. The expression of 81 genes decreased by a factor of 10 or more (Table 4).

Several genes encoding proteins involved in the transport of sugars, acids and amino acids that were expressed at a low level, or were even absent during batch growth, were transcribed in response to glucose limitation. Among them many genes encoding substrate-binding proteins were present. Transport proteins for ribose (RbsD and RbsC), rhamnose (RhaT), arabinose (AraE), lactate (LldP), acetate (ActP), glycerol (GlpF) and glycerol 3-phosphate (UgpA), glucitol and sorbitol (SrlA), mannitol (CmtA), galactitol

(GatC) and C_4 dicarboxylic acids (DctA) were detected with enhanced expression levels. Amino acid transporters such as those for histidine (HisP), glutamate/aspartate (GltL), lysine/arginine/ornithine (ArgT), alanine/serine/glycine (CycA) and peptides (CstA and DppB) were also highly upregulated. Interestingly, the genes of transport proteins that were found to be upregulated most were those for the uptake of acetate (*actP*), peptides (*cstA*) and two members of an operon (*ego* and *yneA*) supposedly involved in the uptake of autoinducer 2 (Wang *et al.*, 2005). Only a few genes of transport systems were downregulated significantly under the conditions tested, and the majority of them are of unknown function.

The physiological response to low glucose concentration led to the upregulation of several genes encoding enzymes of the central metabolism, particularly of enzymes of the TCA pathway and glyoxylate bypass. Among these genes, the highest expression level was found for *prpC*, encoding methylcitrate synthase and belonging to the *prpBCDE* operon. Methylcitrate synthase can take over the function of citrate synthase (GltA) and is known to be transcriptionally regulated by cAMP and σ^N (Lee *et al.*, 2005). An important gene for the TCA cycle, acetyl-CoA synthetase, which converts acetate to acetyl-CoA, was upregulated to significant levels. Surprisingly, fumarate reductase (FrdABCD), which is known to work under anaerobic conditions (Guest, 1981), was also upregulated significantly, whereas transcription of succinate dehydrogenase (SdhABCD), which is negatively regulated by RpoS (Patten *et al.*, 2004), was downregulated. Two enzymes involved in the glyoxylate shunt were affected differently: whereas malate synthase G (GlcB) was upregulated, isocitrate dehydrogenase (IcdA) was downregulated. The glyoxylate shunt has been proposed to play an important role under glucose-limited conditions (Fischer & Sauer, 2003; Wick *et al.*, 2001). In addition, mRNAs of two aldehyde dehydrogenases were found at high levels: AldA, which is known to function on a broad spectrum of substrates (Limon *et al.*,

Fig. 2. Gene classes whose mRNA expression levels ($P \leq 0.05$) were either enhanced by a factor of ≥ 2 (a) or decreased by a factor of ≤ -2 (b), in glucose-limited cultures of *E. coli* MG1655 grown at $D = 0.3 \text{ h}^{-1}$ and harvested from the physiological (short-term) adaptation phase (see Fig. 1).

Table 3. *E. coli* genes whose expression levels were increased by a factor ≥ 5 ($P \leq 0.05$) in cultures exposed for either 40 or 500 h to glucose-limited growth conditions in continuous culture ($D = 0.3 \text{ h}^{-1}$)Genes with an expression factor ≤ 5 but present under one of the two tested conditions are only listed if the *P*-value was ≤ 0.05 .

Gene	b no.	Gene product	Ratio	
			40 h	500 h
Transport				
yajR	b0427	Putative transport protein	5.9	
cusC	b0572	Transport protein involved in copper tolerance	5.7	
cusB	b0574	Putative resistance protein	19.2	
cstA	b0598	Peptide transporter induced by carbon starvation	38.6	
gltL	b0652	ATP-binding protein of glutamate/aspartate transport system	7.1	
ydcS	b1440	Putative transport protein, ABC superfamily	14.8	3.4
ydcU	b1442	Putative transport system permease protein	33.8	
yddP	b1484	Putative ATP-binding component of a transport system	5.9	
ego	b1513	Putative ATP-binding component of a transport system	39.6	16.7
ydhL	b1648	Putative transport protein	5.7	
atoE	b2223	Short-chain fatty acid transporter	6.1	
hisP	b2306	ATP-binding component of histidine transport	7.6	
argT	b2310	Lysine-, arginine-, ornithine-binding periplasmic protein	8.3	
fadL	b2344	Transport of long-chain fatty acids	7.7	
cysA	b2422	ATP-binding component of sulfate permease A protein	5.9	
cysW	b2423	Sulfate transport system permease W protein	5.3	
cysU	b2424	Sulfate, thiosulfate transport system permease T protein	5.6	
cmtA	b2933	PTS system, mannitol-specific enzyme II component	5.8	
ygjU	b3089	Putative transport protein	9.4	
yhdW	b3268	Putative periplasmic binding transport protein	11.6	
ugpC	b3450	ATP-binding protein of sn-glycerol 3-phosphate transport system		2
ugpA	b3452	sn-Glycerol 3-phosphate transport system	5	
dppB	b3543	Dipeptide transport system permease protein 1	11.9	
kup	b3747	Low-affinity potassium transport system	5.2	
rbsD	b3748	D-Ribose high-affinity transport system	7.6	2.9
rbsC	b3750	D-Ribose high-affinity transport system	5.6	
rbsB	b3751	D-Ribose high-affinity transport system		2.7
glpF	b3927	Facilitated diffusion of glycerol	30.8	
actP	b4067	Acetate permease	26.6	3.9
cycA	b4208	Transport of D-alanine, D-serine and glycine	7	
ytfR	b4228	Putative ATP-binding component of a transport system	14.6	
sgcX	b4305	Putative PTS transport system	11.4	
Carbon and energy metabolism				
yahN	b0328	Putative cytochrome subunit of dehydrogenase		9.7
prpC	b0333	Methylcitrate synthase, propionate metabolism	77.9	6
prpD	b0334	Propionate catabolism operon	14.1	
mhpE	b0352	4-Hydroxy-2-ketovalerate aldolase	7.9	
apbA	b0425	Involved in thiamin biosynthesis	5.3	
nadA	b0750	Quinolinate synthetase, A protein	8.4	
galK	b0757	Galactokinase		9
dmsB	b0895	Anaerobic dimethyl sulfoxide reductase subunit B	16.3	
appC	b0978	Probable third cytochrome oxidase, subunit I	6.9	
dadA	b1189	D-Amino acid dehydrogenase subunit	17.4	
aldA	b1415	Aldehyde dehydrogenase, NAD-linked	17.4	4.2
adhP	b1478	Alcohol dehydrogenase	7.2	
fumC	b1611	Fumarase C=fumarate hydratase Class II, isozyme	10.5	
astA	b1747	Arginine succinyltransferase	29	
astC	b1748	Acetylornithine δ-aminotransferase	10.6	
fadD	b1805	Acyl-CoA synthetase, long-chain-fatty-acid-CoA ligase	13.3	

Table 3. cont.

Gene	b no.	Gene product	Ratio	
			40 h	500 h
<i>gatD</i>	b2091	Galactitol-1-phosphate dehydrogenase	7·6	5
<i>gatY</i>	b2096	Tagatose-bisphosphate aldolase 1		
<i>glcB</i>	b2976	Malate synthase G	6·2	
<i>glcF</i>	b2978	Glycolate oxidase iron–sulfur subunit	25·7	
<i>malP</i>	b3417	Maltodextrin phosphorylase	6	
<i>aldB</i>	b3588	Aldehyde dehydrogenase B	31·6	
<i>lldD</i>	b3605	L-Lactate dehydrogenase	5·6	
<i>ilvB</i>	b3671	Acetolactate synthase I, valine-sensitive, large subunit	9	
<i>tnaA</i>	b3708	Tryptophanase	14·7	
<i>fadB</i>	b3846	4-Enzyme protein: 3-hydroxyacyl-CoA dehydrogenase	9·4	
<i>glpK</i>	b3926	Glycerol kinase	16·1	
<i>acs</i>	b4069	Acetyl-CoA synthetase	23·7	
<i>frdD</i>	b4151	Fumarate reductase, anaerobic, membrane anchor polypeptide	24·9	
<i>frdB</i>	b4153	Fumarate reductase, anaerobic, iron–sulfur protein subunit	11·7	
Regulation				
<i>yaiN</i>	b0357	Putative α -helix chain	7·2	
<i>yliH</i>	b0836	Putative receptor	7·4	
<i>ybjJ</i>	b0845	Putative DEOR-type transcriptional regulator	13·8	
<i>ydeW</i>	b1512	Putative transcriptional regulator, SorC family	23·1	10·4
<i>yneA</i>	b1516	Putative LACI-type transcriptional regulator	11·1	4
<i>yeaT</i>	b1799	Putative transcriptional regulator LYSR-type	6·3	
<i>yfaX</i>	b2248	Putative regulator	6·8	
<i>ygeV</i>	b2869	Putative transcriptional regulator	12·7	
<i>dgoR</i>	b3694	Putative FADA-type transcriptional regulator	5	
<i>basR</i>	b4113	Transcriptional regulatory protein, two-component regulatory system	6·3	2·5
<i>adiY</i>	b4116	Putative ARAC-type regulatory protein	5·9	
<i>melR</i>	b4118	Regulator of melibiose operon	11·5	
<i>ytfQ</i>	b4227	Putative LACI-type transcriptional regulator	11·4	
<i>bglJ</i>	b4366	Two-component transcriptional regulator	5·5	
Cell structure				
<i>ycbQ</i>	b0938	Putative fimbrial-like protein	10·9	
<i>csgB</i>	b1041	Minor curlin subunit precursor, similar to CsgA	6·1	
<i>flgM</i>	b1071	Anti-FlhA (anti-sigma) factor	5·4	
<i>flgC</i>	b1074	Flagellar biosynthesis, cell-proximal portion of basal-body rod	12	
<i>flgD</i>	b1075	Flagellar biosynthesis, initiation of hook assembly		4·2
<i>flgE</i>	b1076	Flagellar biosynthesis, hook protein	15·3	
<i>ycjM</i>	b1309	Putative polysaccharide hydrolase	7·9	4·4
Stress response				
<i>fsr</i>	b0479	Fosmidomycin resistance protein	8·4	
<i>cspD</i>	b0880	Cold-shock protein	25·8	
<i>ycbF</i>	b0944	Putative chaperone		6·3
<i>umuC</i>	b1184	SOS mutagenesis and repair	5·7	
<i>pspE</i>	b1308	Phage-shock protein	7·1	2·2
<i>soxS</i>	b4062	Regulation of superoxide response regulon	7·9	11·9
Protein processing				
<i>sixA</i>	b2340	Phosphohistidine phosphatase	6·3	
Amino acid biosynthesis				
<i>cadA</i>	b4131	Lysine decarboxylase 1	5·6	
Nucleotide biosynthesis				
<i>add</i>	b1623	Adenosine deaminase	6·9	
Miscellaneous functions				
<i>fixX</i>	b0044	Putative ferredoxin	5·7	

Table 3. cont.

Gene	b no.	Gene product	Ratio	
			40 h	500 h
<i>rzpR</i>	b1362	Putative Rac prophage endopeptidase	13.7	
<i>yhbL</i>	b3209	Sigma cross-reacting protein 27A (SCR-27A)	8.8	
Unknown or hypothetical functions				
<i>yaiH</i>	b0376	Putative enzyme	5.6	
<i>ybaK</i>	b0481	ORF, hypothetical protein	14.9	6.3
<i>ybhP</i>	b0790	ORF, hypothetical protein	11.5	3.3
<i>yliI</i>	b0837	Putative dehydrogenase	7.9	
<i>ycbN</i>	b0935	ORF, hypothetical protein	7	
<i>ymgE</i>	b1195	ORF, hypothetical protein	5.1	
<i>ychH</i>	b1205	ORF, hypothetical protein	11.1	9.3
<i>ychN</i>	b1219	ORF, hypothetical protein		11.5
<i>ynaK</i>	b1365	ORF, hypothetical protein	6.2	
<i>paaY</i>	b1400	Putative transferase	5	
<i>ydbD</i>	b1407	ORF, hypothetical protein		6.9
<i>ydcH</i>	b1426	ORF, hypothetical protein	8.7	
<i>ydcW</i>	b1444	Putative aldehyde dehydrogenase	8.1	
<i>yncB</i>	b1449	Putative oxidoreductase	5.3	
<i>yncG</i>	b1454	Putative transferase	7.3	
<i>yncH</i>	b1455	ORF, hypothetical protein	7.7	
<i>yneC</i>	b1518	ORF, hypothetical protein	6.8	2.9
<i>ydfU</i>	b1560	ORF, hypothetical protein	9.6	3.4
<i>ynfH</i>	b1590	Putative DMSO reductase anchor subunit	26.9	
<i>ydgD</i>	b1598	ORF, hypothetical protein	7.9	
<i>ydhF</i>	b1647	ORF, hypothetical protein	5.4	
<i>yedP</i>	b1955	ORF, hypothetical protein	5.4	
<i>yedY</i>	b1971	Putative reductase	5.2	
<i>yeeI</i>	b1976	ORF, hypothetical protein	17.8	4.4
<i>yegP</i>	b2080	ORF, hypothetical protein	7	
<i>yohC</i>	b2135	ORF, hypothetical protein	8.7	
<i>yfaE</i>	b2236	ORF, hypothetical protein	5.5	
<i>yfaW</i>	b2247	Putative racemase	7	
<i>yfcX</i>	b2341	Putative enzyme	10.6	
<i>yfeO</i>	b2389	ORF, hypothetical protein	5.9	
<i>ucpA</i>	b2426	Putative oxidoreductase		6.3
<i>ypfE</i>	b2462	ORF, hypothetical protein	7.7	
<i>csiE</i>	b2535	ORF, hypothetical protein	11.3	
<i>ygaD</i>	b2700	ORF, hypothetical protein	5.9	
<i>yqeC</i>	b2876	ORF, hypothetical protein	10.4	
<i>ygfI</i>	b2877	ORF, hypothetical protein	14	
<i>yqfA</i>	b2899	Putative oxidoreductase	7.1	
<i>yghZ</i>	b3001	Putative reductase	19.5	
<i>yglL</i>	b3081	Putative NADPH dehydrogenase	5.8	
<i>yglR</i>	b3087	ORF, hypothetical protein	14.3	
<i>b3122</i>	b3122	ORF, hypothetical protein	9.2	
<i>yhaG</i>	b3128	Putative hydrolase	5.2	
<i>smf_2</i>	b3285	ORF, fragment 2	7.5	
<i>yjbH</i>	b4029	ORF, hypothetical protein	5.1	
<i>yjbR</i>	b4057	ORF, hypothetical protein	6.6	
<i>yjcF</i>	b4066	ORF, hypothetical protein	10.7	
<i>yjcH</i>	b4068	ORF, hypothetical protein	36.8	
<i>yjdA</i>	b4109	Putative vimentin	8.4	
<i>yjfN</i>	b4188	ORF, hypothetical protein	77.1	

Table 3. cont.

Gene	b no.	Gene product	Ratio	
			40 h	500 h
<i>yjfO</i>	b4189	ORF, hypothetical protein	72.6	17.5
<i>yjfY</i>	b4199	ORF, hypothetical protein	5.9	
<i>ytfl</i>	b4215	ORF, hypothetical protein	9.6	
<i>ytff</i>	b4216	ORF, hypothetical protein	45.5	4.1
<i>yjfA</i>	b4223	ORF, hypothetical protein	5.4	
<i>yjjM</i>	b4357	ORF, hypothetical protein	6	
<i>yifM_1</i>	b4404	ORF, conceptual translation in SWISS-PROT is fused with b4405	6.5	

1997), and AldB, which is specific for acetaldehyde and is growth phase-dependent (Ho & Weiner, 2005).

As expected, transcription of genes involved in chemotaxis and the motility machinery were upregulated under glucose-limited conditions, with the purpose to allow cells to move and to search for a better environment. Consistent with this, increased transcription of the chemotaxis regulator (CheY) was found together with that of some periplasmic binding proteins that are also involved in chemotaxis towards the transported carbon sources. Also several genes belonging to class II flagella biosynthesis were upregulated, which is in contrast to a recent transcriptome study where these genes were found to be negatively regulated by RpoS (Patten *et al.*, 2004). The upregulation of genes involved in the flagellar machinery in an *rpoS* mutant might be due to σ competition, where more σ^{70} can bind to RNA polymerases, resulting in housekeeping genes being transcribed at a higher rate. The σ competition can involve also σ^F , which is involved in flagellar machinery expression and shows growth-rate-dependent expression (Makinoshima *et al.*, 2003). At a dilution rate of 0.3 h^{-1} *rpoS* was upregulated fourfold, which is consistent with the fact that the RpoS protein is expressed at half its maximum level under glucose-limited continuous cultures at this dilution rate (Ihssen & Egli, 2004). Many genes reported to belong to the σ^S regulon in other transcriptome studies (Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005) were upregulated under the condition tested. All genes reported earlier to be transcribed in a σ^S -dependent fashion (Patten *et al.*, 2004; Weber *et al.*, 2005) were of hypothetical function, where the most expressed operon of this regulon was the *ycdSTUV*. YdcS was reported to be highly expressed in the phase of physiological adaptation under the conditions tested in the proteome analysis study (Wick *et al.*, 2001). Many genes encoding stress proteins that are not σ^S -dependent were expressed at higher levels in cells grown for 40 h under glucose-limited as compared to batch conditions. One of them is a cold-shock protein (CspD).

Most of the genes encoding proteins belonging to the protein synthesis apparatus and elongation factors were

downregulated. This is consistent with the fact that the specific growth rate of the chemostat culture (0.3 h^{-1}) is only half that in the exponential growth phase in batch cultures (0.63 h^{-1}) (Gourse *et al.*, 1986; Miura *et al.*, 1981). Other operons encoding DNA replication, RNA degradation and purine and pyrimidine nucleotide *de novo* biosynthesis were also negatively affected by the slow growth in continuous culture. Surprisingly, some genes belonging to the virulence group were downregulated as a physiological response to glucose-limited conditions. Also genes encoding proteins involved in the regulation of antigen production, antigen synthesis, haemolysin and colicin production, as well as colicin uptake, were found in this group. Furthermore, several stress proteins were downregulated under the tested condition, such as chaperones, cold- and heat-shock proteins, damage-inducible proteins and resistance proteins; overall, CspF and YdgF were those most affected. Interestingly, some energy-related genes were also downregulated during slow growth in glucose-limited culture, among them cytochrome synthesis proteins, electron transport complexes and the ATP synthesis machinery.

Changes in mRNA levels during the genetic (long-term) adaptation

After 500 h of cultivation in glucose-limited continuous culture, expression of 83 genes was induced at significant levels (≥ 2 , Supplementary Table S2) and the major group found was the one consisting of genes with miscellaneous or hypothetical function (Fig. 3a). Genes that were either expressed with an induction factor >5 , or had a lower induction factor than 5 but were transcribed at high expression levels in the 40 h old culture, are listed in Table 3. In the evolved population 233 genes were downregulated (Supplementary Table S4), with all of them well distributed in the five groups mentioned (Fig. 3b). Genes with expression levels lower than a factor of 10 in long-term adapted cultures of *E. coli* under glucose-limited conditions are listed in Table 4.

With some exceptions, the general pattern detected in cells after a long-term evolution in glucose-limited continuous culture was very similar to that observed for cells

Table 4. *E. coli* genes whose expression levels were decreased by a factor ≤ -10 ($P \leq 0.05$) in cultures exposed for either 40 or 500 h to glucose-limited growth conditions in continuous culture ($D=0.3 \text{ h}^{-1}$)Genes with an expression factor ≥ -10 but present under one of the two tested conditions are only listed if the *P*-value was ≤ 0.05 .

Gene	b no.	Gene product	Ratio	
			40 h	500 h
Transport				
potB	b1125	Spermidine/putrescine transport system permease	−36.2	
potA	b1126	ATP-binding component of spermidine/putrescine transport	−15.7	
ydgF	b1600	Multidrug transport protein	−64.5	
lysP	b2156	Lysine-specific permease	−39.8	
yeiU	b2174	Putative permease	−15.2	
ptsH	b2415	PTS system protein HPr	−4.5	−13.7
exbD	b3005	Uptake of enterochelin, TonB-dependent uptake of B colicins	−11.6	−28.2
secG	b3175	Protein export – membrane protein	−20.3	−20.6
hdeD	b3511	Putative transporter protein		−24.3
yhjV	b3539	Putative transporter protein	−15.4	
mgtA	b4242	Mg ²⁺ transport ATPase, P-type 1	−13.1	
Carbon and energy metabolism				
entE	b0594	2,3-Dihydroxybenzoate-AMP ligase	−16.7	
fldA	b0684	Flavodoxin 1	−4.8	−23.5
sdhC	b0721	Succinate dehydrogenase, cytochrome <i>b</i> ₅₅₆	−13	−19.3
gapC_2	b1416	Glyceraldehyde-3-phosphate dehydrogenase (second fragment)	−6.5	−10.3
gnd	b2029	Gluconate-6-phosphate dehydrogenase, decarboxylating	−4.1	−10.3
serA	b2913	D-3-Phosphoglycerate dehydrogenase	−8.7	−12.4
glnA	b3870	Glutamine synthetase	−36.4	−8.3
Regulation				
hha	b0460	Haemolysin expression modulating protein	−11.3	−13.7
yncE	b1452	Putative receptor	−15	
evgA	b2369	Putative positive transcription regulator (sensor EvgS)	−12	−50
suhB	b2533	Inositol monophosphatase	−20	−3.8
fis	b3261	Site-specific DNA inversion stimulation factor	−16.1	−4.4
yhiW	b3515	Putative ARAC-type regulatory protein	−13.3	
gadX	b3516	Putative ARAC-type regulatory protein	−12.9	−59.5
rnpA	b3704	RNase P	−28.3	−30.9
mioC	b3742	Initiation of chromosome replication	−10.2	−9.8
dnaB	b4052	Replicative DNA helicase, part of primosome	−13.1	
priB	b4201	Primosomal replication protein N	−15.4	−11
fecR	b4292	Regulator for <i>fec</i> operon, periplasmic	−18	−29.9
Cell structure				
ybjE	b0874	Putative surface protein	−23.6	
sfa	b0991	Suppresses <i>fabA</i> and <i>ts</i> growth mutation	−35.1	−57.5
bdm	b1481	Biofilm-dependent modulation protein	−25.5	
wzzB	b2027	Regulator of length of O-antigen component	−13	
spr	b2175	Putative lipoprotein	−13.5	−9.4
cvpA	b2313	Membrane protein required for colicin V production	−26.7	
kdtA	b3633	3-Deoxy-D- <i>manno</i> -octulosonic acid transferase	−14	
yidC	b3705	60 kDa inner-membrane protein	−11.3	
fimA	b4314	Major type 1 subunit fimbrin (pilin)		−10
Stress response				
dacA	b0632	D-Alanyl-D-alanine carboxypeptidase	−9.4	−12
cspG	b0990	Homologue of Salmonella cold shock protein	−8.9	−18
mdoC	b1047	Essential for succinylation of periplasmic glucans	−28.9	
cspF	b1558	Cold-shock protein	−69.4	−51.8
hdeA	b3510	Periplasmic chaperone of acid-denatured proteins		−38.2

Table 4. cont.

Gene	b no.	Gene product	Ratio	
			40 h	500 h
Protein processing				
<i>rpsT</i>	b0023	30S ribosomal subunit protein S20	−21.2	−3.7
<i>ykgM</i>	b0296	Putative ribosomal protein		−27.9
<i>queA</i>	b0405	Synthesis of queuine in tRNA	−13.6	
<i>infA</i>	b0884	Protein chain initiation factor IF-1	−18	−15.5
<i>rpmF</i>	b1089	50S ribosomal subunit protein L32	−10.4	−5.4
<i>yehF</i>	b1203	Putative GTP-binding protein	−16.8	
<i>yehP</i>	b2171	Putative elongation factor	−20.6	
<i>rplY</i>	b2185	50S ribosomal subunit protein L25	−23.7	−23.8
<i>rpsU</i>	b3065	30S ribosomal subunit protein S21	−20.9	−40
<i>rpmA</i>	b3185	50S ribosomal subunit protein L27	−17.6	−15.8
<i>rplU</i>	b3186	50S ribosomal subunit protein L21	−12.4	−11.2
<i>rpsI</i>	b3230	30S ribosomal subunit protein S9	−10.6	−13.8
<i>rplM</i>	b3231	50S ribosomal subunit protein L13	−10.6	−10.1
<i>rpsS</i>	b3316	30S ribosomal subunit protein S19	−10.2	−11.2
<i>rplW</i>	b3318	50S ribosomal subunit protein L23	−7.1	−12.1
<i>rpsJ</i>	b3321	30S ribosomal subunit protein S10	−10.1	
<i>rpmG</i>	b3636	50S ribosomal subunit protein L33	−16.4	−11
<i>rpmB</i>	b3637	50S ribosomal subunit protein L28	−15.7	−13.3
<i>rpmH</i>	b3703	50S ribosomal subunit protein L34	−29.2	−26.9
<i>yihK</i>	b3871	Putative GTP-binding factor	−16.7	−9.3
<i>rplK</i>	b3983	50S ribosomal subunit protein L11	−14.2	
<i>rplA</i>	b3984	50S ribosomal subunit protein L1	−16.8	−17.1
<i>efp</i>	b4147	Elongation factor P (EF-P)	−14.4	−3.6
<i>rpsR</i>	b4202	30S ribosomal subunit protein S18	−12.4	
<i>rplI</i>	b4203	50S ribosomal subunit protein L9	−12.4	−12.4
Amino acid biosynthesis				
<i>speE</i>	b0121	Spermidine synthase = putrescine aminopropyltransferase	−12.5	−20.5
Nucleotide biosynthesis				
<i>carA</i>	b0032	Carbamoyl-phosphate synthetase, glutamine (small) subunit	−22.2	
<i>gpt</i>	b0238	Guanine–hypoxanthine phosphoribosyltransferase	−13	
<i>apt</i>	b0469	Adenine phosphoribosyltransferase	−14.2	
<i>cmk</i>	b0910	Cytidylate kinase	−15.9	
<i>pyrD</i>	b0945	Dihydro-orotate dehydrogenase	−22.9	−58.1
<i>purA</i>	b1232	Formyltetrahydrofolate deformylase	−9.5	−14.4
<i>purC</i>	b2476	Phosphoribosylaminoimidazole-succinocarboxamide synthetase	−25	
<i>purM</i>	b2499	Phosphoribosylaminoimidazole synthetase = AIR synthetase	−31.3	
<i>purN</i>	b2500	Phosphoribosylglycinamide formyltransferase 1	−11.6	
<i>pyrG</i>	b2780	CTP synthetase	−10.4	−5.2
<i>mutM</i>	b3635	Formamidopyrimidine DNA glycosylase	−13.4	
<i>pyrL</i>	b4246	<i>pyrBI</i> operon leader peptide	−13.5	−4.9
Unknown or hypothetical functions				
<i>yagU</i>	b0287	ORF, hypothetical protein	−8.3	−25.6
<i>ybfE</i>	b0685	ORF, hypothetical protein	−10.2	
<i>ybjX</i>	b0877	Putative enzyme	−7.4	−32.6
<i>yccW</i>	b0967	Putative methyltransferase	−17.1	
<i>yedM</i>	b1012	Putative enzyme	−10.2	
<i>yefC</i>	b1132	ORF, hypothetical protein	−14.6	−31
b1354	b1354	Rac prophage	−10.7	
<i>yodA</i>	b1973	ORF, hypothetical protein	−10.6	−13.5
<i>yefL</i>	b2187	ORF, hypothetical protein	−10.6	
<i>yfdI</i>	b2352	Putative ligase	−22.4	

Table 4. cont.

Gene	b no.	Gene product	Ratio	
			40 h	500 h
<i>yfhL</i>	b2562	ORF, hypothetical protein	−22·8	
<i>yqhA</i>	b3002	ORF, hypothetical protein	−14	
<i>ygiR</i>	b3016	ORF, hypothetical protein	−15	
<i>yhdG</i>	b3260	Putative dehydrogenase	−21·2	−28·8
<i>yhiE</i>	b3512	ORF, hypothetical protein		−25·1

collected from the physiological response phase. Several genes involved in transport of glucose and alternative carbon sources were upregulated after growth of *E. coli* cultures for 500 h in the chemostat, but most of these genes were expressed at lower expression factors. A similarly reduced expression of the group of overexpressed genes was obvious for all groups analysed, with one exception, namely the regulator of the superoxide response regulon (SoxS).

In the evolved population also for the downregulated genes the same general pattern (affecting the same gene classes as found in the physiological adaptation) was found, where the same genes were affected with a higher repression factor compared to the physiological response (Table 4). Interestingly, expression levels of the PTS proteins Hpr and PtsI were strongly reduced. These proteins are common to all PTS sugar uptake systems. This downregulation might be a consequence of the expression of a multitude of high-affinity uptake systems, which confer nutritional flexibility to the catabolome.

Expression levels of RpoS were found to be only half of those detected after 40 h under glucose-limited conditions, although proteins (HdeA, HdeD and YhiE) belonging to the σ^S regulon (Patten *et al.*, 2004; Weber *et al.*, 2005) were highly repressed in evolved cells. However, the genes most affected were members of the protein synthesis apparatus

and therefore this effect is probably mainly a consequence of the reduced growth rate.

Real-time RT-PCR

The microarray data were validated using the real-time RT-PCR method. Genes to be confirmed were selected based on the proteome analysis (Wick *et al.*, 2001) and the first microarray data in this study. Generally, the results obtained with this technique confirmed the increase detected with the transcriptome analysis (*mglA*, *malE*, *rhsB*, *dppA* and *katE*) and in the proteome study (Wick *et al.*, 2001). Disagreement was apparent between the microarray and real-time PCR data for six genes (*mglB*, *mglC*, *rpoS*, *lamB*, *aldA* and *ugpB*), for which expression levels were higher with the real-time PCR technique (Fig. 4). Data obtained with the real-time PCR are probably more reliable, because this technology is more sensitive for low amounts of mRNA.

The mRNA extracted from evolved cells confirmed the increased expression pattern obtained with the genome-wide dataset, but compared to the array data, real-time PCR indicated consistently higher induction ratios (Fig. 5). Expression levels of the galactose operon were increased by a factor of 3 between 40 and 500 h in glucose-limited continuous culture, and these results are in good agreement with the proteome analysis (Wick *et al.*, 2001). Some

Fig. 3. Gene classes whose mRNA expression levels ($P \leq 0.05$) were either enhanced by a factor of ≥ 2 (a) or decreased by a factor of ≤ -2 (b), in glucose-limited cultures of *E. coli* MG1655 grown at $D = 0.3 \text{ h}^{-1}$ and harvested from the genetic (long-term) adaptation phase (see Fig. 1).

Fig. 4. Induction factor analysed after 40 h cultivation under glucose-limited conditions compared with the control mRNA (batch culture). Real-time PCR results (white bars) are compared with data obtained from one microarray experiment (black bars) and with proteome results (hatched bars) from Wick *et al.* (2001).

discrepancy was obtained with the RpoS-dependent *katE* gene, which was slightly downregulated in the transcriptome analysis whereas real-time PCR analysis indicated a 10-fold downregulation. Enzyme analysis again showed a decrease of catalase HPII (KatE) specific activity (Visick & Clarke, 1997) that was five times less than that in the starting culture (data not shown).

DISCUSSION

For drinking water microbiologists it is essential to understand the mechanism of adaptation to the conditions

prevailing in drinking water sources and in treated drinking water and it is crucial to know how micro-organisms survive in this nutrient-poor environment. In this study we analysed the transcriptome of an *E. coli* population exposed to low-glucose conditions either for only a few generations, or during long-term to glucose-limited conditions in the chemostat, and compared it to that of cells grown with glucose excess in batch cultures. The transcriptome analysis clearly demonstrated the enhanced transcription of a variety of uptake and catabolic enzymes for substrates not present in the medium. *E. coli* growing under glucose limitation is prepared for the use of

Fig. 5. Induction factor analysed after 500 h cultivation under glucose-limited conditions compared with the control mRNA (batch culture). Real-time PCR results (white bars) are compared with data obtained from one microarray experiment (black bars) and with proteome results (hatched bars) from Wick *et al.* (2001).

alternative carbon substrates and this state is linked also with an upregulation of various periplasmic high-affinity binding proteins. Our results are in agreement with the high-affinity uptake system expression reported previously for glucose-limited continuous cultures (Ihssen & Egli, 2005; Wick *et al.*, 2001). This pattern of protein expression under glucose limitation is not specific for this sugar only, and was reported also for carbon-limited growth in continuous culture with other sugars and complex medium (Ihssen & Egli, 2005), and even for batch growth with unfavourable carbon substrates supporting only low specific growth rates (Liu *et al.*, 2005).

Carbon/energy-limited growth at suboptimal rates, and not the presence of an appropriate substrate, seems to be the major factor for the high expression of different uptake systems (Ihssen & Egli, 2005; Liu *et al.*, 2005; Wick *et al.*, 2001). Cultures of *E. coli* growing under glucose-limited conditions also express mRNAs for enzymes of central metabolic and respiratory pathways to elevated levels (Hua *et al.*, 2003). The same pattern was observed also with the respiration rates for different substrates (Ihssen & Egli, 2005). This upregulation of genes belonging to transport systems, specific breakdown enzymes and enzymes that facilitate the electron transport from substrate to terminal electron acceptors might be a consequence of the decreased expression of rRNA due to the lower growth rate (Gourse *et al.*, 1986; Kurland & Maaloe, 1962; Liu *et al.*, 2005; Miura *et al.*, 1981). The transcriptome data obtained in this study fit with the catabolic derepression reported earlier using either different 'global' techniques, or focusing only on specific genes/proteins under glucose-limited conditions, such as glucose-limited chemostat and fed-batch conditions (Hua *et al.*, 2004; Notley & Ferenci, 1995; Wick *et al.*, 2001; Yoon *et al.*, 2003). Our results concur with the proteome data and periplasmic protein analysis under glucose-limited continuous culture, where DppA, MalE, MglB and RbsB expression levels are upregulated (Ihssen & Egli, 2005; Wick *et al.*, 2001). These and other transporter proteins expressed here at high levels were reported to be under the control of cAMP (Gosset *et al.*, 2004; Salgado *et al.*, 2004; Tan *et al.*, 2001).

The most important global regulator in the adaptation to low carbon and energy sources seems to be cAMP. This global regulator induces most high-affinity transport systems, as well as many starvation proteins (Matin *et al.*, 1989). The cAMP concentration is high in cells growing in glucose-limited chemostat cultures, whereas under glucose-excess conditions cAMP concentrations are low (Notley-McRobb *et al.*, 1997). The derepression of ABC transporters is caused by a combination of increased cAMP concentration and induction by specific sugar endoinducers (Ferenci, 1999a; Tweeddale *et al.*, 1998). For example, the maltose and galactose operon are induced under glucose-limited conditions by the production of intracellular maltotriose (Notley & Ferenci, 1995) and galactose (Ferenci, 1996), respectively.

The expression pattern found during the short-term response, also referred to as physiological adaptation, was observed as well after the long-term (genetic) adaptation to glucose (carbon/energy) limitation. These findings are in agreement with the 2D gel electrophoresis analysis performed under the same conditions (Wick *et al.*, 2001) and catabolome analysis using Biolog as reported by Ihssen & Egli (2005) recently (data not shown). In populations of *E. coli* evolved under glucose-limited conditions, fewer genes were found with increased or decreased expression.

In addition to the induction of alternative transport systems, the carbon and energy metabolism under glucose-limited conditions was affected seriously. Proteome (Wick *et al.*, 2001) and carbon fluxes analysis (Fischer & Sauer, 2003) indicated the presence of a new metabolic cycle, the PEP-glyoxylate cycle, that appears to increase growth efficiency under glucose-limited conditions. The PEP-glyoxylate cycle, which was known to work only in the presence of acetate or fatty acids (Cronan & LaPorte, 1996), might confer more metabolic flexibility to the cells (Ihssen & Egli, 2005). Isocitrate lyase (AceA) and isocitrate dehydrogenase (IDH) are two key enzymes required for this bypass during growth on acetate or fatty acids as sole carbon and energy sources (Cronan & LaPorte, 1996). IDH is known to be regulated by IDH kinase/phosphatase (AceK), which phosphorylates and inactivates IDH (Koshland *et al.*, 1985). In our study, IDH inactivation was monitored as downregulation of its expression and not by an upregulation of AceK, which is involved in the inactivation of IDH. The second key enzyme of the glyoxylate shunt, isocitrate lyase, was strongly expressed under glucose-limited and fed-batch conditions (Hua *et al.*, 2004; Wick *et al.*, 2001; Yoon *et al.*, 2003), but not in our experiment.

Interestingly, we found some upregulated genes of the citrate cycle that were not induced in previous studies (Hua *et al.*, 2004; Yoon *et al.*, 2003). Methylcitrate synthase, which was the most expressed gene during the physiological adaptation, is thought to take over the function of citrate synthase to produce citrate. The proteins making up fumarate reductase, which is known to be expressed under anaerobic conditions (Cronan & LaPorte, 1996), are very similar to those of succinate dehydrogenase, which acts under aerobic conditions, and they can take over the function of each other (Guest, 1981). Fumarate reductase produces high amounts of superoxide at low succinate concentrations, whereas superoxide formation is low at enhanced concentrations of succinate (Cecchini *et al.*, 2002). Hence, the PEP-glyoxylate cycle, switched on under glucose-limited conditions, produces high concentrations of succinate, and this again might reduce superoxide production by fumarate reductase. An alternative explanation is linked to the expression of RpoS; fumarate reductase expression is positively controlled by σ^s whereas succinate dehydrogenase expression is negatively regulated by this sigma factor (Weber *et al.*, 2005). Many genes belonging to

the TCA cycle, except those of the *sucCD* operon, are down-regulated by RpoS (Patten *et al.*, 2004).

These observations might explain overexpression in our microarray data of those genes that are not under negative control of the stress response sigma factor. During the first 40 h in glucose-limited continuous culture at a dilution rate of 0.3 h^{-1} RpoS is present in high amounts (Ihssen & Egli, 2004) and hence no genes belonging to this regulon were downregulated. However, evolved cells carried an RpoS attenuation phenotype and as a consequence a large part of the σ^s -dependent genes were downregulated. The expression level of *rpoS* was partially reduced in 500-h-old cells, but the protein translation and stability might be affected also, resulting in the attenuated phenotype. It is well known that the synthesis of RpoS is not limited to gene transcription, but it is affected at all levels starting from mRNA stability via translation, to protein stability and proteolysis (Hengge-Aronis, 2002). The attenuation or the deletion of RpoS was monitored under glucose-limited conditions (Notley-McRobb *et al.*, 2002) and in prolonged cultivation of stationary-phase cells (Farrell & Finkel, 2003). The phenotype resulting from this attenuation or deletion of RpoS exhibited a higher $\mu_{\text{max, Glc}}$ and an improved K_s, Glc compared to the wild-type strain (Wick *et al.*, 2002). *rpoS* mutant strains arising under carbon-limited conditions possess a growth advantage and these strains are strongly selected for under such conditions (Notley-McRobb *et al.*, 2002). This phenomenon has also been observed in batch stationary-phase cultures, where they have been referred to as GASP (Growth Advantage in Stationary Phase) mutants (Farrell & Finkel, 2003; Notley-McRobb *et al.*, 2002; Zambrano & Kolter, 1996). Genetic polymorphism in the *rpoS* genomic region is common in different *E. coli* populations (Ferenci, 2003). High levels of σ^s and negative effects on transporter expression reduce the competitiveness of the strain in low-glucose environments (King *et al.*, 2004). The attenuation or deletion of σ^s reduces the competition between this sigma factor and σ^{70} (Farrell & Finkel, 2003; Nyström, 2004), which is necessary for an enhanced transcription of genes needed for the uptake and utilization of carbon sources.

The mRNA pools are highly dynamic, and one of the major problems for microarray analysis is to collect mRNA from cells in the same physiological state during independent repeats. Therefore, the experimental design is very important using transcriptome analysis, where a multitude of results are obtained that must be reproduced and validated. Although batch cultures are very easy to perform, they are problematic with respect to sample reproducibility. The requirement for reproducibility can be met by using continuous culture, where cells are in a so-called steady-state.

The main findings of our study were the catabolic flexibility of *E. coli* and a switch from the TCA pathway to the PEP-glyoxylate cycle in glucose-limited continuous culture. Moreover, this catabolic flexibility and induction of the

PEP-glyoxylate cycle were observed also after genetic selection under such conditions. A downregulation of *rpoS* transcription and of genes belonging to the σ^s regulon was observed in the evolved population, increasing the importance of cAMP as global regulator during adaptation to carbon/energy limitations.

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