Short- and long-term changes in proteome composition and kinetic properties in a culture of *Escherichia coli* during transition from glucose-excess to glucose-limited growth conditions in continuous culture and vice versa

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Summary

To investigate the ability of *Escherichia coli* K12 MG1655 to cope with excess and limitation of a carbon and energy source, we studied the changes in kinetic properties and two-dimensional (2D) gel protein patterns of an *E. coli* culture. The population was transferred from glucose-excess batch to glucose-limited continuous culture (D = 0.3 h⁻¹), in which it was cultivated for 500 h (217 generations) and then transferred back to glucose-excess batch culture. Two different stages to glucose-limitation were recognized: a short-term physiological adaptation characterized by a general effort in enhancing the cell’s substrate scavenging ability and mutations resulting in a population exhibiting increased glucose affinity. Physiological short-term adaptation to glucose-limitation was achieved by upregulation of 12 proteins, namely MglB, MalE, ArgT, DppA, RbsB, YdcS, LivJ (precursor), UgpB (precursor), AceA, AldA, AtpA and GatY. Eight of these proteins are periplasmic binding proteins of ABC transporters. Most of them are not involved in glucose transport regulons, but rather in chemotaxis and transport of other substrates, whereas MalE and MglB have previously been shown to belong to transport systems important in glucose transport under glucose-limited conditions. Evolution under low glucose concentration led to an up to 10-fold increase in glucose affinity (from a Kₐ of 366 ± 36 μg l⁻¹ at the beginning to 44 ± 7 μg l⁻¹). The protein pattern of a ‘500-h-old’ continuous culture showed a highly increased expression of MglB and MalE as well as of the regulator protein MalI. When adapted cells taken from the ‘500-h-old’ continuous culture were transferred to batch culture, an increased expression of MalE was observed, compared with cells from unadapted batch-grown cells. Otherwise, no significant changes were observed in the protein pattern of batch-grown populations before and after 500 h of evolution in the glucose-limited continuous culture.

Introduction

*Escherichia coli* is usually not considered to be a typical environmental bacterium. However, this bacterium is present in considerable number in many ecosystems (Savageau, 1983), particularly in warm climate countries, in which it not only survives but can also grow (Hazen and Toranzos, 1990). Throughout the world, the presence of *E. coli* is used as an indication for faecal contamination of surface water and drinking water, and therefore knowledge of its behaviour under nutrient-limited conditions in the aqueous environment is crucial.

In principle, non-sporulating bacteria such as *E. coli* can encounter three growth situations. Growth at saturating substrate concentrations (probably exclusively in the laboratory), growth at subsaturating substrate concentrations and non-growth. Growth at saturating substrate concentrations and non-growing bacteria have been studied extensively (Groat *et al*., 1986; Schultz *et al*., 1988; Kolter *et al*., 1993; Weichart *et al*., 1993; Hengge-Aronis, 1996; 2000). However, starvation experiments have strong drawbacks: they are difficult to perform reproducibly and ill-defined with respect to the cellular status (see Discussion in Ferenci, 1999). Moreover, for a considerable part of their lifetime, bacteria in the environment are neither growing at their maximum rate nor are they completely starved, but they grow at reduced rates under nutrient-limited conditions. In most ecosystems the limiting factor is the availability of organic compounds, which
results in a limitation in carbon and energy sources. This led to the proposition that a carbon-limited continuous culture is the most appropriate experimental tool to investigate the behaviour of bacterial growth under environmental conditions (Matin, 1979; Moriarty, 1993; Morita, 1993).

Studies concerning the global response of cells to carbon-limited conditions have revealed a number of different physiological phenomena; however, the molecular mechanisms involved here are still poorly investigated. Certain carbon sources that lead to a two-step growth pattern under carbon-excess conditions can be used simultaneously by cells grown under carbon-limited conditions (e.g. in batch cultures containing lactose and glucose, *E. coli* first uses glucose, and lactose is consumed only after the depletion of glucose, whereas in carbon-limited continuous cultures the two sugars are used simultaneously (Silver and Mateles, 1969)). Such cells also exhibit a higher activity of several catabolic enzymes, even in the absence of the appropriate carbon sources and are able to utilize a wide range of sugars without an induction lag (Matin, 1979; Harder and Dijkhuizen, 1983; Sepers, 1984; Lendenmann and Egli, 1995).

Indications for a general improvement of glucose affinity as a result of exposure to low glucose concentrations were reported for a number of bacteria (marine isolates: Jannasch, 1968; *E. coli*: Shehata and Marr, 1971; Koch and Wang, 1982; *Klebsiella pneumoniae*: Rutgers et al., 1987; *Cytophaga johnsonii*: Höflle, 1983). For *Cytophaga johnsonii* (Höflle, 1983) and for *E. coli* ML30 (Senn et al., 1994), the time course of this improvement has been documented.

Several aspects of the glucose-specific evolution of *E. coli* in glucose-limited continuous cultures have been studied (Helling et al., 1987; Kurlandzka et al., 1991; Rosenzweig et al., 1994; Senn et al., 1994; Ferenci, 1996; Notley-McRobb and Ferenci, 1999a,b). Ferenci (1996) has shown two regulons to be important in glucose uptake: *mal/lam* and *mgl/gal*. Under glucose-limited conditions, *E. coli* enhances the outer membrane permeability for glucose (and other sugars) by higher expression of *lamB* and uses the high affinity ABC transport system *mgl* for glucose uptake through the inner membrane. As an immediate physiological response to glucose-limited conditions, these systems are upregulated by endo-induction (e.g. the induction of the *mal* operon by the endogenously synthesized inducer maltolose during growth on glucose) and cAMP, whose levels are increased in continuous culture-grown *E. coli* compared with cells grown under glucose-excess conditions (Ferenci, 1996; Notley-McRobb et al., 1997). During long-term cultivation in continuous culture, the population evolves and mutations in three regulatory loci (*mlc*, *mglD/O* and *malT*) are selected that lead to higher expression of the *mal/lam* regulon and the *mgl* regulon. These mutations result in a better affinity for glucose (Notley-McRobb and Ferenci, 1999a,b).

In this work we investigated the kinetic and physiological behaviour of *E. coli* during the transition from glucose-excess to glucose-limited growth conditions in the continuous culture. In particular, the changes occurring at the proteome level were studied using two-dimensional (2-D) gel electrophoresis.

**Results**

**Evolution of kinetic properties of E. coli in glucose-limited continuous culture**

During long-term cultivation of *E. coli* in a glucose-limited continuous culture at a fixed dilution rate, a continuous improvement of the culture’s glucose scavenging ability with time was observed, resulting in a steadily decreasing residual glucose concentration. The residual glucose concentration only became (apparently) stable after some 400 h and this improvement of the Monod-K*ₙ* was highly reproducible between different runs (Fig. 1).

In addition to the decrease of the residual glucose concentration in the continuous culture, cells that were taken from the ‘500-h-old’ (adapted) continuous culture and transferred to batch culture with an initial glucose concentration of 500 mg l⁻¹ showed a higher $\mu_{\text{max}}$ ($0.73 \pm 0.01$ h⁻¹) than cells from the ‘40-h-old’ continuous culture (0.63 ± 0.01 h⁻¹). The apparent Kₙ of the culture can be calculated based on the modified (including a $s_{\text{min}}$ value) Monod model of microbial growth: $\mu = \mu_{\text{max}} \times (s - s_{\text{min}})/ (K_{\text{g}} + s - s_{\text{min}})$, in which $\mu_{\text{max}}$ is the maximum specific growth rate determined in batch culture, $\mu_{\text{g}} (= D = 0.3 \pm 0.015$ h⁻¹) is the specific growth rate given at the substrate concentration s, Kₙ is the substrate concentration that allows growth at half the maximum specific growth rate, and $s_{\text{min}}$ (12 ± 2 μg l⁻¹) is the minimally needed substrate concentration for growth (Kovarova et al., 1996). The data in Fig. 1 suggest that, during the 500 h of cultivation (corresponding to 217 generations), the Kₙ of the culture improved from the

![Fig. 1. Time-course of the residual glucose concentration in glucose-limited continuous cultures of E. coli K12 operated at D = 0.3 h⁻¹. Data for four independent runs are shown.](image-url)
initially \(366 \pm 36 \mu g \text{ l}^{-1}\) down to \(44 \pm 7 \mu g \text{ l}^{-1}\) (average s-values in the initial 72 h of four independent continuous cultivations were \(345 \pm 21 \mu g \text{ l}^{-1}\), those between 455 and 535 h of three independent continuous cultivations were \(43 \pm 4 \mu g \text{ l}^{-1}\)). These \(K_v\) values correlate very well with the 10-fold increase in glucose affinity reported by Notley-McRobb and Ferenci (1999b) for derivatives of E. coli K12 after long-term exposure to glucose-limited growth in continuous culture.

**Proteome analysis of cells grown under different growth conditions using 2-D gel analysis**

Cells that had been growing in the glucose-limited continuous culture for different periods of time (40, 156 and 500 h) and from exponentially growing batch cultures, before and after exposure to glucose-limited continuous culture conditions, were sampled. From each condition three to four individual samples were collected from two different continuous culture runs. Cells were harvested and subjected to proteome analysis using 2-D gel electrophoresis. Protein spots whose intensity was found to vary by a factor of three or more between these different conditions were cut off and the protein material was analysed by mass spectrometry after in-gel digestion. The masses of proteolytic peptides were used to perform Peptide Mass Fingerprinting (Henzel et al., 1993; James et al., 1993; Mann et al., 1993; Pappin et al., 1993; Cottrell, 1994). In several cases the identification was confirmed by tandem mass spectrometry followed by database searches with raw mass spectrometry MS/MS spectra (Yates et al., 1995). The results for the protein identification are summarized in Table 1.

**Proteome changes during the transfer from glucose-excess batch to glucose-limited continuous culture conditions.** Figure 2 shows the 2-D gels of cells harvested in the log phase of the initial batch culture and cells after 40 h in continuous culture. A comparison of the gels in Fig. 2 reveals a number of differently expressed proteins. The spots that differed between the two growth conditions are numbered in Fig. 2 and listed in Table 1. It is striking that the majority of the increasingly expressed proteins were periplasmic binding proteins of different transport systems. Although glucose was the only energy and carbon source in the medium, also binding proteins that are not involved in glucose transport were upregulated under the glucose-limited conditions compared with the glucose-excess batch conditions. No spots could be detected with significantly lower intensities in cells taken from the continuous culture compared with the batch-grown cells (note that integrated quantities for every spot were normalized over the total optical density (OD) of every gel and are expressed as percentages).

**Proteome changes during evolution in glucose-limited continuous cultivation.** The proteins that were found to be up/downregulated during prolonged cultivation in glucose-limited conditions are shown in Fig. 3. Most impressive is the amount to which the two binding proteins (MalE and MglB, spot no. 8 and 9, respectively, Figs 2 and 3) contributed to the total gel protein (together \(\sim 30\%\)). Another impressive upregulation was observed for MalI (spot no. 13, Fig. 3), a putative regulator protein, a member of a class of proteins that are normally expressed at very low levels.

**Batch growth of cells cultivated for 500 h in a glucose-limited continuous culture**

Cells taken from the ‘500-h-old’ continuous culture (1 ml) were used to inoculate a shaking flask containing

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>pI</th>
<th>mw</th>
<th>Protein (gene)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.07</td>
<td>52.1</td>
<td>Aldehyde dehydrogenase A (aldA)</td>
<td>Central metabolism</td>
</tr>
<tr>
<td>2</td>
<td>5.75</td>
<td>57.4</td>
<td>Periplasmic dipeptide transport protein (dppA)</td>
<td>Peptide transport</td>
</tr>
<tr>
<td>3A</td>
<td>5.16</td>
<td>47.5</td>
<td>Isocitrate lyase (aceA)</td>
<td>Central metabolism</td>
</tr>
<tr>
<td>3B</td>
<td>5.16</td>
<td>47.5</td>
<td>Isocitrate lyase (aceA)</td>
<td>Central metabolism</td>
</tr>
<tr>
<td>4</td>
<td>5.98</td>
<td>46.1</td>
<td>Glycerol-3-phosphate-binding periplasmic protein (ugpB)</td>
<td>Transport</td>
</tr>
<tr>
<td>5</td>
<td>6.02</td>
<td>55.2</td>
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<td>Proton transport/energy</td>
</tr>
<tr>
<td>6</td>
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<td>39.9</td>
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<td>7</td>
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<td>Maltose-binding periplasmic protein (malE)</td>
<td>Sugar transport</td>
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<td>5.25</td>
<td>33.6</td>
<td>D-Galactose-binding periplasmic protein (mglB)</td>
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<td>5.98</td>
<td>31.1</td>
<td>Tagatose-bisphosphate aldolase (gatY)</td>
<td>Catabolism</td>
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<td>6.46</td>
<td>36.8</td>
<td>Maltose regulon, regulatory protein (malI)</td>
<td>Sugar transport</td>
</tr>
</tbody>
</table>

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**Table 1. Proteins analyzed in this study.**

a. The cellular amount of proteins differed at a factor of three or more between cells grown in a glucose-excess-batch and in a glucose-limited continuous culture (1–12, see also Fig. 2), and between cells from a ‘40-h-old’ and a ‘500-h-old’ continuous culture (3B, 7, 8, 9, 11, 12, 13, see also Fig. 3).

b. Molecular weight and pI as calculated from the sequence. Values were found to be consistent (\(\pm 0.15\) pI units) with migration on 2-D gels.

0.5 g l\(^{-1}\) of glucose. These cells were kept at \(\mu_{\text{max}}\) for about 30 generations and were then used to inoculate again a bioreactor. This population exhibited a higher \(\mu_{\text{max}}\) (0.73 \(\pm\) 0.01 h\(^{-1}\)) than the population taken from the stock and initially used (0.63 \(\pm\) 0.01 h\(^{-1}\)). Again cells were harvested for 2-D gel analysis. %Vol. values of MalE were found to increase from 0.101 \(\pm\) 0.025 to 0.928 \(\pm\) 0.232 in cells collected from the ‘500-h-old’ continuous culture compared with the population at the start (Fig. 4, lanes 1 and 2).

To determine the apparent \(K_s\) of these cells, the bioreactor was switched to continuous cultivation mode, as described previously, at D = 0.3 h\(^{-1}\) and the residual glucose concentration was determined. Two hours after the biomass had reached steady-state concentration the residual glucose concentration was 122 \(\mu\)g l\(^{-1}\), indicating a \(K_s\) of 158 \(\mu\)g l\(^{-1}\). This demonstrates that the population of long-term glucose-adapted \(E. coli\) – when re-grown in batch culture – still exhibited an improved affinity for glucose.

The percentages of total gel protein of each of the proteins listed in Table 1, obtained from cells grown under the different conditions, are shown in Fig. 4. After 500 h of cultivation in the continuous culture, most proteins that are not involved in glucose transport were found in lower cellular relative amounts compared with cells from a 40 h-old chemostat. This, however, is mainly a consequence of the enormous increase of the two proteins MalE and MglB (from about 7% to 30%), resulting in a dilution of all other proteins. As the spots of MalE and MglB are saturated, this value is probably still an underestimation.

DppA and GatY are exceptions, because their amount was also increased in cells cultivated for an extended time.
in the glucose-limited continuous culture. In the case of DppA, one can speculate that higher amounts of this dipeptide-binding protein are used in recycling peptides otherwise lost into the medium.

Identification of mutants

As MalE itself has only a negligible affinity to glucose (Kellermann and Szmelcman, 1974), an overexpression of malE does not seem to be advantageous under glucose-limited conditions. Therefore, it was appealing to assume that the higher MalE levels are an indication of mal/lamB constitutivity, which was previously shown to be of advantage in glucose-limited cultures (Notley-McRobb and Ferenci, 1999a). Therefore, a sample from a '500-h-old' continuous culture was diluted appropriately, plated on nutrient agar and nine colonies were picked randomly. These clones were analysed for their sensitivity towards methyl-α-glucoside and 2-deoxyglucose to test for mlc mutations. Two clones showed higher sensitivity towards 2-deoxyglucose, one of these also to methyl-α-glucoside. In addition, sequencing of the malT gene (coding for a regulatory protein of the mal operon) was performed. Three of the other seven clones, which did not show increased sensitivity to the glucose analogues, had point mutations in malT: M311I ATG→ATA (in two clones) and R268H CGC→CAC. The former mutation was also found in populations evolved under glucose-limited conditions analysed by Notley-McRobb and Ferenci (1999a). Hence, five of the nine clones showed regulatory mutations affecting the mal operon including malE.

Discussion

We have investigated here the response of E. coli to the transition between two environments that are distinctly different with respect to the availability of the carbon/energy source. The best approach to analyse this response experimentally seems to be the comparison of cells grown under glucose-excess conditions in batch
culture and glucose-limited conditions in a continuous culture at a set growth (dilution) rate. Thereby, it is inevitable that other physiological parameters will also change, the most obvious being the growth rate. Except in the case of AtpA, there were no hints for growth-rate-dependent differences, such as e.g. the variation of the ribosomal protein concentrations. However, the data of Maaloe (1979) suggest that distinct physiological changes resulting from a variation in growth rate occur only at elevated growth rates (1 h\(^{-1}\) to 3 h\(^{-1}\)) and should not be observed in our study, in which the cells experienced a change in specific growth rate from 0.6 h\(^{-1}\) to 0.7 h\(^{-1}\) in batch to 0.3 h\(^{-1}\) in the continuous culture. Our results indicate that the response of the cells to glucose-limited conditions can be divided into two distinct phases, namely a short-term physiological adaptation and a long-term selection of a population with increased affinity to glucose.

The following discussion will focus on the difference between these short- and long-term answers. Although the 2-D gel technique allows us to get a global picture of a cell’s response to distinct environmental stimuli, this method allows, at best, detection of approximately 50% of the proteins expressed. A rough estimate for this study indicates that we were able to detect about 20% of the total proteins. This means that it is very unlikely that complete sets of proteins belonging to a pathway or regulon will be found. For example, two proteins that have been shown to be upregulated under glucose-limited conditions, namely LamB and OmpF (Death et al., 1993; Liu and Ferenci, 1998), were not detected in this study. In the following discussion we will therefore take the results obtained as hints to pathways and regulons activated by the conditions we applied to the cells.

**Effects during short-term adaptation**

Eight of the 12 proteins whose expression was significantly different in cells grown under glucose-excess and in cells under glucose-limited conditions are binding proteins, and, hence, are transport related. Two proteins are involved in catabolism (AldA, GatY), one (AceA) in the central metabolism (citric acid cycle/glyoxylate bypass) and the other (AtpA) in energy generation.

**Binding proteins.** We observed enhanced expression of seven out of the approximately 20 binding proteins belonging to uptake systems for C- and C/N-sources (carbohydrates and amino acids) (Linton and Higgins, 1998; Paulsen, 1999). Included are all the binding proteins involved in chemotaxis, i.e. MglB, MalE, RbsB, DppA (Oliver, 1996). Additionally, one putative ABC binding protein was found (YdcS). The 2-D gel electrophoresis technique does not allow us to draw conclusions about the behaviour of the remaining binding proteins involved in uptake of alternative carbon sources that were not detected in this study. One should keep in mind that the expression of ABC transport systems is regulated by different and rather complex mechanisms (Boos and Lucht, 1996; Ehrmann et al., 1998), and carbon-limitation is probably only one of several elements involved in the control of binding protein expression.

Our results clearly underline the importance of ABC uptake systems for growth in the environment and this is also supported by the fact that, in *E. coli*, the family of ABC transporter genes occupies about 5% of the whole genome (Linton and Higgins, 1998). It was suggested that the binding protein is largely responsible for the apparent affinity of the transport system for its substrate (Miller et al., 1983). The fact that the increase in the apparent affinity for glucose from 366 μg l\(^{-1}\) to 44 μg l\(^{-1}\) is paralleled by an increase in the binding protein responsible for glucose transport (MglB) is additional strong evidence for this hypothesis.

From the two binding proteins LivJ and UgpB, only the precursors and not the mature forms were found on the 2-D gels. This may be as a result of the overproduction of other secreted periplasmic proteins (especially MalE and MglB) leading to competition for signal peptidase.

**Enzymes involved in catabolism and central metabolism.**

The two enzymes GatY and AldA, the expression of which was enhanced in the early phase of adaptation, are enzymes of catabolic pathways. GatY catalyses the cleavage of tagatose-1,6-bisphosphate to gliceraldehyde-3-phosphate and dihydroxyacetone-phosphate; hence, it connects the galactitol degradation to the glycolytic pathway. It was also found to be induced at low pH-values (Blankenhorn et al., 1999). AldA is an aldehyde dehydrogenase known to function on a broad spectrum of substrates and to respond to multiple regulatory signals, one of them being cAMP-CRP (Limon et al., 1997).

The isocitrate lyase as the first and most abundant enzyme in the glyoxylate bypass is normally considered to be induced only when *E. coli* is growing on acetate or fatty acids, and its function is to replenish the dicarboxylic acids consumed in amino acid biosynthesis. During growth on glucose, however, the glyoxylate pathway is not required as the anaplerotic reaction is performed through the PEP-pathway (Cronan and LaPorte, 1996). It cannot be deduced from our data whether or not there is any flux of carbon through the glyoxylate bypass, as this depends on phosphorylation of IDH by IDH kinase/phosphatase (Koshland et al., 1985). However, the net-reaction of both pathways (2 pyruvate + oxaloacetate—2 oxaloacetate + 2CO\(_2\)) yields 4 NADH, 1 FADH\(_2\), but in the PEP-pathway one molecule of ATP is consumed in addition. Thus, the balance of the two anaplerotic sequences suggests the glyoxylate pathway to be more
economical, a fact that might become important under carbon-limited conditions. An additional reason for the enhanced level of isocitrate lyase could be that, in this way, the cell is able to respond more quickly to a sudden availability of C2-generating substrates such as acetate and fatty acids.

Two spots on the gels (Fig. 2. spot no. 3 A and 3B) were identified as AceA. A possible explanation could be that the protein occurs in different covalently modified forms, although no evidence was found earlier for covalent modification of AceA (Kosslhand et al., 1985). In addition, proteolysis cannot be ruled out.

AtpA. The atpBEFHA operon encodes the genes for the F0F1 proton-translocating ATPase, which couples the energy derived from oxygen respiration to ATP synthesis and the ATPase alpha chain constitutes a regulatory subunit of this enzyme complex. Kasimoglu et al. (1996) showed that atp gene expression is inversely related to the growth rate. This agrees well with our finding of an increased expression of AtpA under the glucose-limited conditions performed at a D of 0.3 h⁻¹, whereas growth under glucose-excess conditions results in a Dₘₐₓ more than twice as high.

Regulatory network aspects. At least six of the genes found upregulated under glucose-limited conditions in this work show either a dependency on cAMP-CRP or have a putative CRP binding site. These are malE (Ullmann and Danchin, 1983), mglB, rbsB (Alexander et al., 1993), ugpB (Su et al., 1991), argT (Botsford and Harman, 1992) and aldA (Limon et al., 1997). In E. coli, stimulation of the cAMP-CRP regulon is well known to be an immediate response to glucose limitation aiming at the use of alternative carbon sources (Saier et al., 1996). Furthermore, Schultz et al. (1988) found that, from the 30 proteins induced within the first 4–5 h of carbon starvation, 20 of them were not induced in a Δcrp or Δcrp strain. It has recently been confirmed that cAMP levels of E. coli cells grown in continuous culture at μM glucose concentrations are enhanced compared with those grown under glucose-excess conditions (Notley-McRobb et al., 1997). However, for many genes controlled by cAMP-CRP, cAMP is not the only regulating signal (Blum et al., 1990). For example, in the case of the ABC maltose transporter, the regulation of the expression of its genes is far more complex than originally expected (Ehrmann et al., 1998; Boos and Böhm, 2000).

Long-term evolution

MalE and MglB. These two binding proteins, belonging to the mgl and mal regulon, were the two most abundant proteins on the 2-D gels of cells from the ‘500-h-old’ glucose-limited cultures. The binding protein MglB has a high affinity for glucose (Ferenci, 1996), higher than that of the PTS-system (Postma et al., 1993), and, at low glucose concentrations, glucose is taken up by the cells mainly via this transport system. The fact that this sugar enters the cells mainly via the (high affinity) galactose transport system during growth at low glucose concentrations was visualized earlier by pulsing galactose into a steady-state glucose-limited continuous culture of E. coli (Egli et al., 1993). A pulse of 1.8 mg l⁻¹ of galactose resulted in an immediate reduction of the glucose utilization rate of the cells and led to a transient accumulation of glucose in the continuous culture. Hence, the proteome analysis performed in this work suggests that the same changes take place during the evolution of the population as those reported by Notley-McRobb and Ferenci (1999a;b) who approached the question from the genetic side.

MalI. A strikingly high amount of the regulatory protein MalI was found in cells harvested from the ‘500-h-old’ continuous culture. It should be pointed out that such an increased expression of a regulatory protein might have a much broader effect than a similarly increased expression of a catabolic enzyme like GatY. MalI is a repressor of an operon containing malX and malY. MalI is not dependent on MalT (Boos and Shuman, 1998), thus excluding the possibility that the increased levels of MalI might be an effect of malT-con, as in the case of MalE. The gene malX is an enzyme II of the phosphotransferase system recognizing an as yet unidentified substrate. This operon might thus become important under glucose-limited conditions, in which transport systems of alternative carbon sources are generally expressed at higher levels. The physiological role of MalY is still obscure. It is supposed to either act on the substrate transported by MalX or to interact with MalT, when the carbon source transported by MalX is available. In any case, MalY binds MalT reversibly, stabilizing the inactive monomeric form of MalT and in this way interferes with its transcriptional activator function (Clausen et al., 2000). Therefore, MalY is not desired when high MalT activity is required. However, malT-con mutants are largely insensitive to the effect of MalY (Boos and Shuman, 1998), making the role of MalI in this context quite puzzling.

Pleiotropic effects. In our experiments, pleiotropic effects on the protein expression pattern were detected only during the short-term adaptation but not as a result of long-term evolution of the culture. The long-term effects observed were all geared to an improved uptake of glucose. This is in contrast to the findings of Kurlandzka et al. (1991) who reported that, for cells kept in glucose-limited continuous culture for a period of 773 generations,
changes in the protein expression pattern included a broad spectrum of cellular functions. There are several reasons to explain these differences. In our study, the culture was analysed as a whole, whereas Kurlandzka et al. (1991) analysed single clones isolated from their culture. Furthermore, after sampling they included a labelling step with radioactive methionine. In addition, dilution rate (0.2 vs. 0.3 h\(^{-1}\)), temperature (30 vs. 37°C) and time of evolution (773 vs. 217 generations) were different. Therefore, it is probably difficult to compare the two sets of data. Growth at lower growth rates and lower temperatures is paralleled with higher RpoS and CspA levels, respectively (Notley and Ferenci, 1996; Phadtare et al., 2000), thus enhancing the probabilities for mutations with pleiotropic effects. The most crucial difference, however, is the one in cultivation time. The study by Ferenci (1996) and this work have shown that, in the first 200–300 generations of glucose-limited growth, the bacteria gain strong fitness advantages by improving their glucose-scavenging ability through some easily occurring mutations. After this (the largest) potential has been exploited, further evolution probably involves genes with pleiotropic effects, as here considerable improvements can also be achieved with few mutations.

**Effect of long-term evolution under glucose-limited conditions to growth during glucose-excess conditions**

There are several studies that show that mutants with increased \(\mu_{\text{max}}\) can be isolated from cultures that had been exposed to prolonged continuous cultivation (Dykhuizen and Hartl, 1981; Helling et al., 1987; Weikert et al., 1997). In addition, in this study a population with a higher \(\mu_{\text{max}}\) than the starting culture was enriched from the glucose-limited cultivation. This culture exhibited a higher amount of MalE protein under glucose-excess conditions than the starting population (lane 1 and 2 in Fig. 4), which can be explained by assuming the mal operon to still be under less tight control than in wild-type cells. A higher \(v_{\text{max}}\) would be the result of such a process. However, a higher transport rate should not be the reason for the higher \(\mu_{\text{max}}\) observed for these cells, as transport rate is not considered to be the limiting process for determining \(\mu_{\text{max}}\) (Holmes, 1996).

**Conclusion**

In the short term, i.e. within the first hours of limited glucose availability, the cells respond to glucose limitation by making a general effort to increase the ability to scavenge and utilize different carbon/energy substrates. It should be pointed out that the synthesis of high-affinity uptake systems and increased amounts of metabolic enzymes are not only restricted to enzymes involved in the metabolism of the supplied growth-limiting carbon source glucose, but were also observed for a range of other carbon/energy substrates that were not supplied in the growth medium. This ability to utilize mixtures of carbon sources simultaneously and the readiness to use carbon sources immediately when they become available is of high importance for survival and competition ability under environmental conditions, in which the spectrum of available carbon/energy sources is large and not restricted to a single one (Egli, 1995). Using this strategy, the cells not only gain an advantage in a better exploitation of the diversity of nutrients in an ecosystem but are also prepared for the immediate utilization of nutrients that might become suddenly available in their environment, thus getting a head start compared with unprepared organisms. Hence, our findings at the proteome level are a good basis to explain the phenomenon of mixed substrate growth at the molecular level observed earlier under carbon-limited growth conditions (Matin, 1979; Harder and Dijkhuizen, 1983; Sepers, 1984; Lendenmann and Egli, 1995).

In the long-term, the population evolved specifically as a response to the nutrient stress applied (glucose-limited conditions in this case). Compared with the observed short-term adaptation, which would make sense in nature, this long-term adaptation with its massive upregulation of only a few proteins important in glucose transport is probably unlikely to be found under the growth conditions prevailing in ecosystems, as the selection pressure applied here (growth with only glucose for 217 generations) appears to be rather artificial.

**Experimental procedures**

**Organism and medium**

Wild-type *Escherichia coli* K12 MG 1655 (from the culture collection of P. Postma, Amsterdam) was grown at 37°C in mineral medium containing the following components: 20 mM KH\(_2\)PO\(_4\), 14 mM NH\(_4\)Cl, 0.22 mM EDTA, 0.23 mM MgSO\(_4\), 0.01 mM Na\(_2\)MoO\(_4\), 0.1 mM CaCO\(_3\), 0.0075 mM FeCl\(_3\), 0.025 mM MnCl\(_2\), 0.0125 mM ZnO, 0.005 mM CuCl\(_2\), 0.005 mM CoCl\(_2\) and 0.005 mM H\(_2\)BO\(_3\). Glucose was used as sole source of carbon and energy at concentrations of 0.5 g l\(^{-1}\) and 1 g l\(^{-1}\) for batch conditions and at a concentration of 0.1 g l\(^{-1}\) for continuous cultures, resulting in a glucose-limited medium supporting a \(\mu_{\text{max}}\) of 0.63 ± 0.01 h\(^{-1}\) for the wild-type strain. Nutrient agar and ECD agar MUG, an agar allowing the identification of *E. coli*, were obtained from Biolife.

**Chemicals and biochemicals**

Hen egg lysozyme was purchased from Fluka, while RNase A, DNase and Pefabloc SC were obtained from Boehringer Mannheim. Resolytes 4–8 were purchased from BDH.
Laboratory Supplies. IPG strips were obtained from Amersham Pharmacia Biotech. All other chemicals were of the highest purity available from Fluka. Sequencing grade-modified trypsin was from Promega.

Shake flask cultures

Stock cultures were plated on nutrient agar twice and, from the second plate, a single colony was cultured to inoculate a shake flask containing minimal medium with 0.5 g l\(^{-1}\) of glucose and Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) (72 mM/22 mM) to buffer the system at pH 7. The cells were pregrown for approximately 50 generations under excess glucose conditions. To keep the bacteria constantly growing at \(v_{\text{max}}\), all cells were always transferred into fresh medium before they reached the late log phase. The resulting culture was used to inoculate a bioreactor.

Cultivation in the bioreactor

Batch and continuous culturing of cells was performed in large scale bioreactors (MBR) with a working volume of 1.5 l. The aeration was at a rate sufficient to ensure always > 90% air saturation, impeller speed was set at 1000 r.p.m., and the pH was maintained at 7 ± 0.05 by automatic addition of 0.5 M KOH/0.5 M NaOH.

Batch cultivation. Using cells pregrown in shake flasks, the bioreactor was inoculated to an OD_{546} of 0.015, in the presence of 1 g l\(^{-1}\) glucose. When the cells reached an OD_{546} of 0.4 (early mid-log phase), 1.2 l of the culture was harvested for the subsequent 2-D gel analysis by pouring the cultivation liquid directly in centrifuge tubes containing approximately 30% (v/v) of ice. The reactor was refilled with fresh medium containing 0.1 g l\(^{-1}\) glucose, drained and refilled again. The cells remaining in the reactor served as the inoculum to start the subsequent glucose-limited continuous culture. The resulting initial OD_{546} was approximately 0.08.

Cultivation in glucose-limited continuous culture. When the cells inoculated in a reactor as described reached an OD_{546} of 0.09, the medium flow (containing 0.1 g l\(^{-1}\) of glucose) was started at a dilution rate of 0.3 h\(^{-1}\). The culture was then operated in the continuous mode for 500 h. The purity of the continuous culture was tested by plating onto nutrient agar and ECD agar MUG. Additionally, the culture was checked directly using fluorescence in situ hybridization (FISH) with a Cy3-labelled probe targeting 16S rRNA (Microsynth). Based on the report of Fuchs et al. (1998), potential sequences with low evolutionary conservation and high fluorescence intensities were checked for highest specificity at the webpage of the Ribosomal Database Project from Michigan State University (Maidak et al., 2001) and the following sequence proved as the most suitable: 5'-acttacctcctcctcctc-3'.

Sampling for 2-D gel analysis

Samples were poured directly from the continuous culture into 50 ml Falcon tubes containing 15–20 ml ice, to quickly cool the samples. Cells were harvested by centrifugation for 10 min at 3500 g at 4°C. The cell pellets were suspended and transferred to Eppendorf tubes using ice-cold low-salt buffer (3 mM KCl, 68 mM NaCl, 10 mM Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\), pH 7). Cells were pelleted again in a cooled microfuge and stored at -20°C until gel electrophoresis was performed.

Glucose analysis

Glucose concentrations in continuous cultures were determined as described previously (Senn et al., 1994).

Sample preparation for 2-D gel electrophoresis

Aliquots of frozen E. coli cells, containing approx. 10^{10} cells each, were thawed slowly on ice and resuspended in 250 g l\(^{-1}\) of low-salt buffer (3 mM KCl, 68 mM NaCl, 10 mM Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\), pH 7). After centrifugation for 5 min at 5000 g, the pellet was resuspended in 80 g l\(^{-1}\) of lysis buffer (10 mM Tris/HCl pH 7.5, 1.5 mM MgCl\(_2\), 10 mM KCl, 0.5 mM 1,4-dithio-DL-threitol, 0.5 mM Pefabloc SC) and 5 g l\(^{-1}\) of a hen egg lysozyme solution (10 μg g\(^{-1}\) l\(^{-1}\)) were added. The samples were incubated for 20 min at 37°C and subsequently sonicated for 2 × 10 s with a tip sonicator. To each sample 3 μl of a RNase A solution (10 μg g\(^{-1}\) l\(^{-1}\)) and 5 μl of a DNase solution (10 μg g\(^{-1}\) l\(^{-1}\)) were added and the samples were incubated for 15 min at room temperature to perform digestion of nucleic acids. To each sample 500 μl of solubilization buffer (containing 0.9 M Urea, 4% (w/v) 3-((3-cholamidopropyl)dimethyl-ammonio)-1-propanesulphonate (CHAPS), 65 mM 1,4-dithio-DL-threitol, 0.8% Resolutes 4–8, 0.01% (w/v) bromophenol blue) were then added. After mixing for 1 min, solubilization was allowed to proceed for 15 min at room temperature. Solid urea was added to each tube until saturation was reached, the samples were then centrifuged at 13 000 g for 30 min and the supernatants were used for 2-D gel electrophoresis.

2-D gel electrophoresis

Gels were run in batches of 20 to ensure reproducibility. Samples (500 μl) were used to rehydrate overnight 1 × 18 cm immobiline strips pl 4–7. Isoelectric focusing was performed at a maximum voltage of 3500 V, until a V × h count of 70 000 was reached. Equilibration and transfer to the second dimension were done as described (Hochstrasser et al., 1988). SDS–polyacrylamide gel electrophoresis (PAGE) was performed on 12% acrylamide gels using the Iso-Dalt system (Large Scale Biology Corp.), and Coomassie blue protein staining was according to Schagger and von Jagow (1987).

Image analysis

Gels were scanned on a Personal Densitometer (Molecular Dynamics). Image files were analysed using MELANIE II 2-D PAGE, Version 2.2 (Bio Rad). The spot’s density normalized to the overall density of the gel proteins (referred to as %Vol. values) was taken to compare corresponding protein spots between different gels. Measurement errors were determined
the following way: the intensities of 34 protein spots, which were regarded as constant throughout all five conditions, were quantified and the standard deviation for each was calculated. The spots were then classified into three categories: those with %Vol. values between 0.03 and 0.1, those between 0.1 and 1, and those higher than 1. The average percentage standard deviations for each category were 30%, 25% and 21% respectively.

**In-gel proteolysis and extraction**

Coomassie blue-stained protein spots of interest were excised and washed for 20 min in double-distilled water followed by destaining in 30% acetonitrile, 100 mM ammonium bicarbonate pH 8.0 for 30 min at 37°C under agitation. The excised gel slices were dried in a rotary evaporator and re-hydrated with 20 µl of 100 mM ammonium bicarbonate pH 8.0 containing 0.3 µg of sequencing-grade modified trypsin. Digestion was carried out overnight at room temperature. Peptides were extracted by washing the gels once with 150 µl of double distilled water and twice with 250 µl of 40% acetonitrile, 0.1% trifluoroacetic acid under agitation.

**MALDI-TOF mass spectrometry (MS) analysis**

Peptide extracts from protein spots were pooled, dried and redissolved in 4 µl of 0.2% trifluoroacetic acid. Aliqots of the re-dissolved extracts (0.3 µl) were mixed with an equal volume of α-hydroxycinnamic acid (20 µg µl⁻¹ in 50% acetonitrile) and spotted on the MALDI target plate. After drying, the sample crystals were washed twice with 5 µl of ice-cold 0.1% trifluoroacetic acid and dried again. MALDI-TOF spectra were acquired on a Perseptive Biosystems Voyager Elite mass spectrometer operated in reflector mode. Samples were analysed in delayed extraction reflector mode using an accelerating voltage of 20 kV, a pulse delay time of 150 ns, a grid voltage of 60% and a guide wire voltage of 0.05%. Spectra were accumulated for 32 laser shots.

**ESI-MS/MS analysis**

Samples for electrospray MS analysis were desalted on a capillary column packed with 0.2 µl of POROS R2 resin as described (Wilm et al., 1996). MS/MS sequencing was performed on a Finnigan MAT LC-Q ion trap mass spectrometer. The desalted digest in 70% methanol and 1% acetic acid was loaded into a homemade nanospray tip and electrosprayed into the mass spectrometer at a flow rate of 0.2 µl min⁻¹ using a syringe pump. Peptide ions were manually selected for fragmentation, which was carried out using a relative collision energy of 50–80 units for MH⁻ ions and 20–35 units for MH²⁺ ions.

**Database searches**

MALDI-TOF spectra were examined manually to extract the monoisotopic molecular weights and the obtained sets of masses were used to search *E. coli* protein sequences in the SWISSPROT database using the program PEPTIDENT (available on-line on the site http://www.expasy.ch). Only sequences matching for at least three peptides resulting from complete digestion were taken into account, together with information on the molecular weight and the pl of the protein as derived from the gel.

Raw MS/MS spectra were used to search with the program SEQUEST (Yates et al., 1995), an *E. coli* protein sequence database compiled in-house. Only sequences that were matched by at least two distinct peptide searches with ∆Cn values greater than 0.15 were considered as a positive identification.

**Identification of mlc and malT mutations**

Sequencing of the malT gene and sensitivity tests towards the PtsG and PtsM substrates, methyl-α-glucoside and 2-deoxyglucose, respectively, were performed as described in Notley-McRobb and Ferenci (1999a,b).

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**References**


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