Determination of protein synthesis on a proteomic scale
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Proteome-wide alterations in *E. coli* translation rates upon anaerobiosis
Chapter 5

**INTRODUCTION**

An integrated view of the molecular events underlying adaptation of bacteria to major environmental changes requires insight into both transcriptional and post-transcriptional regulation of gene expression. With the availability of annotated genome databases, much has been learned about global changes induced in mRNA levels and the transcription factors involved, as well as about changes in steady state protein levels upon a switch in environmental conditions. However, little is known concerning genome-wide changes in protein synthesis and degradation-rates and about the contributions of transcription and translation to the regulation of gene expression when bacteria adapt to major changes in the environment. An important reason for this is the fact that it is much easier, using genome-wide microarray analysis, to find candidate genes with expression levels being regulated via RNA transcription or degradation, than to identify candidate genes with expression levels regulated at the level of translation.

Renewed interest in gene expression regulation at the post-transcriptional level in prokaryotes has been sparked recently by the discovery in 2001-2002 of the existence of large numbers of small regulatory RNAs (sRNAs) in *E. coli* and other bacteria (248-251). These sRNAs may regulate translation of numerous mRNAs (61), often mediated by the RNA chaperone Hfq (252). Although the function of many sRNAs is not yet known, several recently reviewed findings (62) strongly suggest that post-transcriptional regulation by sRNAs is widespread and that the number of mRNAs regulated by sRNAs amply exceeds the number of sRNAs themselves. However, not much is known about how environmental signals are transduced to sRNA mediated regulation of translation (57). Identifying potential target genes is an important step in unravelling underlying molecular mechanisms of translational regulation.

What is needed is a proteomic method to determine alterations in translation rates during adaptation to environmental changes. By comparison of changes in translation rates with changes in mRNA levels genes may be identified that are regulated at the translational level. Recently we have developed a pulse-labelling technique using the methionine analogue azhal to determine the relative amounts of proteins synthesized by *E. coli* in a brief time frame upon a change in environmental conditions. Here we show that measuring changes in total protein levels on the same time-scale as new protein synthesis allows identification of stable and labile proteins. We demonstrate that altered levels of most newly synthesized proteins are the result of a change in translation rate rather than degradation-rate after a switch from aerobiosis to anaerobiosis. The majority of proteins with increased synthesis-rates upon an anaerobic switch are involved in glycolysis and pathways aimed at preventing glycolysis grinding to a halt by a cellular redox-imbalance. Our method can also be used to compare relative translation rates with relative mRNA levels. Discrepancies between these parameters may reveal genes whose expression is regulated by translation. This may help unravelling molecular mechanism underlying regulation of translation, e.g. mediated by small regulatory RNAs.

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**SUMMARY**

Enzyme repurposing in bacteria during adaptation from one environmental condition to another may be regulated both by transcription and translation. However, little is known about the contribution of translational regulation. Recently we have developed a pulse-labelling method using the methionine analogue azhal to determine the relative amounts of proteins synthesized by *E. coli* in a brief time frame upon a change in environmental conditions. Here we show that measuring changes in total protein levels on the same time-scale as new protein synthesis allows identification of stable and labile proteins. We demonstrate that altered levels of most newly synthesized proteins are the result of a change in translation rate rather than degradation-rate after a switch from aerobiosis to anaerobiosis. The majority of proteins with increased synthesis-rates upon an anaerobic switch are involved in glycolysis and pathways aimed at preventing glycolysis grinding to a halt by a cellular redox-imbalance. Our method can also be used to compare relative translation rates with relative mRNA levels. Discrepancies between these parameters may reveal genes whose expression is regulated by translation. This may help unravelling molecular mechanism underlying regulation of translation, e.g. mediated by small regulatory RNAs.
degradation of stable newly synthesized proteins during a 10 minutes pulse is negligible, increased or decreased newly synthesized amounts of stable proteins is predominantly the result of a change in translation rate rather than in degradation-rate.

In this study we use the extended azhal pulse-labelling approach and quantitative mass spectrometry to determine, on a proteomic scale, changes in both newly synthesized proteins as well as total protein levels in exponentially growing E. coli cells during their initial adaptation to a sudden drop in oxygen levels. By comparison of changes in newly formed proteins to changes in total protein levels in the initial 10 minutes following the anaerobic switch, stable and labile proteins are identified. For the large group of stable proteins the relative changes in levels of newly synthesized proteins directly reflect the average relative translation rates of these proteins over the pulse-labelling time used. We obtained a dataset of relative translation rates that is consistently related to the required metabolic adaptation, underscoring the reliability of our method.

RESULTS

Growth, labelling and quantitation of newly synthesized proteins upon an anaerobic switch—

E. coli is a facultative anaerobic prokaryote and its ability to switch between aerobic and anaerobic environments greatly expands the range of niches it can thrive in. To enable growth in both environments, a set of metabolic routes for each has to be maintained. Changes that occur in the central carbon metabolism at the onset of anaerobiosis are related to the need for alternative ways to maintain a proper intracellular redox-balance, since molecular oxygen, the terminal electron acceptor of the aerobic electron transport chain, is no longer available to remove the excess reducing equivalents formed in catabolism. This can be achieved by anaerobic respiration, using one or more alternative terminal electron acceptors (253). If no alternative electron acceptor is available, growth on glucose requires removal of reducing equivalents by mixed acid fermentation (254). When grown in aerobic batch cultures, with glucose as energy source and in the absence of alternative electron acceptors, E. coli stops growing upon a switch to anaerobiosis for about 20 minutes and then resumes growth at a slower pace (124). Under these conditions the flux through the phosphotransferase system (PTS) for glucose transport annex phosphorylation and through glycolysis is dramatically increased (255). To study immediate changes in protein synthesis upon an anaerobic switch, methionine-auxotrophic cells grown aerobically in an amino acid supplemented glucose-containing medium were harvested, washed with the same medium without methionine and transferred to either an aerobic or anaerobic environment and pulse-labelled for 10 minutes with azhal. During the pulse of azhal, aerobically cultured cells continue growth and increase their cellular mass by ~6% in 10 minutes, judged from an increase in optical density at 600 nm, while the anaerobic cells stop growing, in agreement with earlier observations (124).

Proteins extracted from azhal-labelled cultures were digested and peptides were labelled with iTRAQ for quantitation. Subsequently, azhal-containing peptides were enriched by COFRADIC, and identified and quantified by LC tandem-MS as outlined in experimental procedures and previously described (141). A total of 414 azhal-containing peptides were identified, corresponding to 211 different proteins synthesized during the pulse-labelling period. From the 211 newly synthesized proteins, 164 could be quantified using the iTRAQ reporter ions, according to the criteria referred to in experimental procedures. Upon a switch to an anaerobic environment the relative abundance of 69 newly synthesized proteins significantly (p<0.05) increased or decreased more than twofold. In addition 31 proteins changed significantly (p<0.05) by a factor of 1.5-2. The relative abundance of the remaining 64 newly synthesized proteins changed less than 1.5 fold or did not change significantly at all during the 10 minutes after the anoxic switch (Figure 1).
Chapter 5 Alterations in translation rates upon anaerobiosis

Pathways affected in the rate of protein synthesis by a sudden change to an anaerobic environment— The change to an anaerobic environment necessitates either activation of the anaerobic respiration machinery or of fermentation pathways. Five alternative terminal reductases can be expressed in *E. coli* (253). We detected newly synthesized subunits from four of these, nitrite-reductase (NirB), DMSO-reductase (DmsA), nitrate reductase (NarG) and fumarate-reductase (FrdA), all being increased at least five-fold. It is noteworthy that the synthesis of NirB, DmsA and NarG rapidly increases while their respective substrates are not present in the environment. The relative new formation under anaerobic conditions of two proteins (MoaB and MoaE) involved in the biogenesis of a molybdenum-cofactor was also increased. This cofactor is part of different molybdenum-containing enzymes, amongst which are the anaerobic respiratory enzymes TMAO-reductase, nitrate-reductase and DMSO-reductase (256). Furthermore we find increased amounts of newly synthesized cytochrome-d oxidase (CyDA) and a putative quinone oxidoreductase (YdhD) (257). The cytochrome-d oxidase is known to be expressed under conditions with low oxygen tensions (258), while increased synthesis of the putative quinone oxidoreductase suggests a function in anaerobic respiration.

Apart from anaerobic respiration, *E. coli* can maintain its redox balance under anaerobic conditions via oxidation of reducing equivalents by mixed acid fermentation (Figure 1a, black dots and Table I). Pyruvate formate lyase (PFL), together with its auxiliary glycyl radical cofactor (GrcA) (259), plays a central role in this respect as it supplies pyruvate dehydrogenase (PDH) in converting pyruvate. PFL catalyzes the formation of formate and acetyl-CoA from pyruvate. Thus, PFL generates both a fermentation end-product and a precursor which can be converted to acetate in a reaction coupled to formation of ATP, or undergo further fermentation to ethanol. Not surprisingly, it was found that the amounts of newly synthesized PflB and GrcA are highly increased upon the anaerobic switch. Concordantly, two major enzymes that regenerate NAD+ from NADH in fermentation are upregulated in synthesis during the first ten minutes following anaerobic switching. AckA, involved in the conversion of acetyl-CoA to acetate, is only modestly up-regulated during the initial response to an anaerobic environment. So, the relative levels of four newly synthesized enzymes (PflB, GrcA, AdhE and LdhA) out of the six enzymes involved in the conversion of pyruvate to formate, acetate, ethanol and lactate were increased at least fourfold, while one (AckA) was increased slightly and the other one (Pta) was not observed. It is also noteworthy that levels of the newly synthesized PDH complex subunits AceE, AceF and LpA hardly change upon anaerobiosis, even though PDH activity is strongly inhibited under anaerobic conditions (255).

Another major pathway that is affected by a switch to anaerobic growth in the absence of alternative electron acceptors is glycolysis. It is the pathway that is responsible

### Table I

*Identity of newly synthesized proteins most elevated 10 minutes after a switch to anaerobiosis in *E. coli.*

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Gene</th>
<th>Ratio†</th>
<th>S.D.</th>
<th>Ratio‡</th>
<th>S.D.</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite reductase [NAD(P)H] large subunit</td>
<td>nirB</td>
<td>20.6</td>
<td>4.7</td>
<td>n.d.</td>
<td>*</td>
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<tr>
<td>Anaerobic dimethyl sulfoxide reductase</td>
<td>dmsA</td>
<td>11.2</td>
<td>4.2</td>
<td>n.d.</td>
<td>*</td>
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<tr>
<td>Aldehyde dehydrogenase</td>
<td>adhE</td>
<td>10.3</td>
<td>2.9</td>
<td>2.6 0.68</td>
<td>θ</td>
<td></td>
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<tr>
<td>Pyruvate formate-lyase</td>
<td>pfB</td>
<td>7.8</td>
<td>4.6</td>
<td>2.3 0.73</td>
<td>θ</td>
<td></td>
</tr>
<tr>
<td>Universal stress protein D</td>
<td>ydiT</td>
<td>6.8</td>
<td>1.8</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Universal stress protein A</td>
<td>yjaA</td>
<td>6.4</td>
<td>1.9</td>
<td>1.5 0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autonomous glycyl radical cofactor</td>
<td>grcA</td>
<td>6.3</td>
<td>2.7</td>
<td>5.4 1.6</td>
<td>θ</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde dehydrogenase</td>
<td>gldA</td>
<td>5.9</td>
<td>0.72</td>
<td>n.d.</td>
<td></td>
<td></td>
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<td>Universal stress protein G</td>
<td>ybdQ</td>
<td>5.9</td>
<td>3.1</td>
<td>n.d.</td>
<td></td>
<td></td>
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<tr>
<td>Respiratory nitrate reductase</td>
<td>narG</td>
<td>5.8</td>
<td>3.1</td>
<td>n.d.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Ketol-acid reducto isomerase</td>
<td>ilvC</td>
<td>5.8</td>
<td>1.5</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fumarate reductase flavoprotein subunit</td>
<td>frdA</td>
<td>5.4</td>
<td>2.2</td>
<td>n.d.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Ribosome-associated inhibitor A</td>
<td>raiA</td>
<td>5.4</td>
<td>1.3</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Phosphofructokinase isozyme I</td>
<td>pfkA</td>
<td>5.2</td>
<td>1.5</td>
<td>1.8 0.27</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Putative quinone oxidoreductase ydhH</td>
<td>ydhH</td>
<td>5.1</td>
<td>0.27</td>
<td>n.d.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Fructose-specific phosphotransferase II</td>
<td>frdA</td>
<td>5.0</td>
<td>1.7</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-alpha-hydroxydesmityl dehydrogenase</td>
<td>dadA</td>
<td>5.0</td>
<td>0.61</td>
<td>0.95 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-lactate dehydrogenase</td>
<td>ldhA</td>
<td>4.4</td>
<td>1.1</td>
<td>n.d.</td>
<td>θ</td>
<td></td>
</tr>
<tr>
<td>Phosphoglyceromutase</td>
<td>gpm</td>
<td>4.3</td>
<td>1.0</td>
<td>1.4 0.10</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Bacterioferritin</td>
<td>btf</td>
<td>4.2</td>
<td>1.0</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probable sigma (54) modulation protein</td>
<td>ybdH</td>
<td>4.0</td>
<td>1.4</td>
<td>n.d.</td>
<td></td>
<td></td>
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<tr>
<td>Malate synthase G</td>
<td>gldC</td>
<td>3.8</td>
<td>0.42</td>
<td>n.d.</td>
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<tr>
<td>Malate dehydrogenase protein B</td>
<td>moaB</td>
<td>3.8</td>
<td>0.57</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome d ubiquinol oxidase subunit 1</td>
<td>cydA</td>
<td>3.4</td>
<td>0.59</td>
<td>1.7 0.24</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Small heat shock protein ipiB</td>
<td>ipIB</td>
<td>3.3</td>
<td>0.26</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>pgk</td>
<td>3.2</td>
<td>0.94</td>
<td>1.3 0.21</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase II</td>
<td>pykA</td>
<td>3.1</td>
<td>0.87</td>
<td>1.2 0.16</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate isomerase</td>
<td>pgi</td>
<td>3.0</td>
<td>0.43</td>
<td>1.2 0.38</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Molybdopterin-converting factor subunit 2</td>
<td>mdeA</td>
<td>2.8</td>
<td>0.41</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPP2 protein yetX</td>
<td>yebX</td>
<td>2.6</td>
<td>0.25</td>
<td>1.4 0.30</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Phosphohomupyrurate-proteinphosphotransferase</td>
<td>yphT</td>
<td>2.5</td>
<td>0.55</td>
<td>1.1 0.13</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase A</td>
<td>gapA</td>
<td>2.5</td>
<td>0.64</td>
<td>1.2 0.16</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Emolase</td>
<td>eno</td>
<td>2.4</td>
<td>0.80</td>
<td>1.1 0.21</td>
<td>*</td>
<td></td>
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<tr>
<td>6-Phosphofructolactonase</td>
<td>pfL</td>
<td>2.1</td>
<td>0.24</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase class 2</td>
<td>fbaA</td>
<td>2.1</td>
<td>0.14</td>
<td>1.1 0.13</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Threonyl-tRNA synthetase</td>
<td>thrS</td>
<td>2.0</td>
<td>0.32</td>
<td>1.1 0.18</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

†, ratio of the amounts of proteins newly synthesized for 10 minutes upon the onset of anaerobiosis and under aerobic conditions determined by quantitation of azhal-containing peptides; †, ratio of the total protein levels (pre-existing plus newly synthesized) 10 minutes after the onset of anaerobiosis and under aerobic conditions determined by quantitation of peptides lacking both azhal and methionine; * proteins involved in anaerobic respiration; θ proteins involved in fermentation; • glycolytic enzymes and PTS sugar transport proteins; n.d., not determined.
for the generation of most energy from glucose under these conditions. Figure 1b (grey diamonds) shows that the relative amounts of the 9 detected glycolytic enzymes synthesized during the first ten minutes after the anaerobic switch increase between 1.6 and 5.2 fold (Table I). This represents ~ 90% of the entire pathway, the only glycolytic enzyme not observed being triose isomerase. Our results corroborate and greatly extend previous observations regarding increases in levels of newly synthesized glycolytic and mixed acid fermentation enzymes (124).

In addition, we observe an increase in the amounts of newly synthesized glucose-specific (PtgA) and fructose-specific (PtfaH) components as well as the phosphoenolpyruvate-protein phosphotransferase (PtsI) part of the PTS which is involved in sugar import and its subsequent phosphorylation prior to entering glycolysis (Figure 1b, grey diamonds and Table I). The PTS protein HPt is lacking in our dataset, probably because tryptic digestion will only yield one methionine-containing peptide. This peptide of 6 amino acids, including the protein C-terminus, can easily escape detection. We did also not detect one other PTS protein, PtsG, a membrane protein responsible for transport and concomitant phosphorylation of glucose.

Despite the absence of some proteins in our dataset, these results indicate that the relative amount of most if not all newly synthesized members of the entire PTS and glycolysis have increased considerably 10 minutes after the onset of anaerobiosis.

The synthesis of three uniform stress proteins (260) i.e. uspD, uspA and uspG was found to increase about six-fold upon anaerobiosis. Two ribosome-associated factors, RaiA and YhbH, were also produced in much higher amounts under anaerobiosis. These two proteins are also found in cells in stationary phase (261) and their upregulation may be related to the transient growth arrest during adaptation to the anaerobic state.

Because transient growth arrest occurs in the anaerobic environment, the overall protein synthesis-rate is expected to become lower under these conditions. Indeed, the levels of all 20 detected newly synthesized ribosomal proteins (out of total of 56), the expression of which is strongly related to growth rate (262, 263), were significantly lower in the anaerobic cells (dark grey triangles in Figure 1b and Table II). Because of their abundance, the synthesis-rates of ribosomal proteins contribute considerably to the overall protein synthesis-rate. Despite the absence of some proteins in our dataset, these results indicate that the relative amount of most if not all newly synthesized members of the entire PTS and glycolysis have increased considerably 10 minutes after the onset of anaerobiosis.

Because transient growth arrest occurs in the anaerobic environment, the overall protein synthesis-rate is expected to become lower under these conditions. Indeed, the levels of all 20 detected newly synthesized ribosomal proteins (out of total of 56), the expression of which is strongly related to growth rate (262, 263), were significantly lower in the anaerobic cells (dark grey triangles in Figure 1b and Table II). Because of their abundance, the synthesis-rates of ribosomal proteins contribute considerably to the overall protein synthesis-rate. Overall, both the proteins found to be up-regulated as well as those found to be down-regulated immediately following the anoxic switch are related to the imposed change in environment. This functional consistency underscores the reliability of our dataset.

<table>
<thead>
<tr>
<th>protein name</th>
<th>gene name</th>
<th>ratio†</th>
<th>S.D.</th>
<th>ratio‡</th>
<th>S.D.</th>
<th>pathway</th>
</tr>
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<tr>
<td>50S ribosomal protein L1</td>
<td>rplK</td>
<td>0.21</td>
<td>0.06</td>
<td>0.95</td>
<td>0.27</td>
<td>#</td>
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<td>50S ribosomal protein L4</td>
<td>rplD</td>
<td>0.26</td>
<td>0.05</td>
<td>0.86</td>
<td>0.16</td>
<td>#</td>
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<td>30S ribosomal protein S8</td>
<td>rpsH</td>
<td>0.26</td>
<td>0.01</td>
<td>0.91</td>
<td>0.07</td>
<td>#</td>
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<tr>
<td>30S ribosomal protein S10</td>
<td>rpsJ</td>
<td>0.26</td>
<td>0.07</td>
<td>0.92</td>
<td>0.17</td>
<td>#</td>
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<tr>
<td>50S ribosomal protein L2</td>
<td>rplB</td>
<td>0.28</td>
<td>0.10</td>
<td>0.86</td>
<td>0.13</td>
<td>#</td>
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<td>cysteine synthase A</td>
<td>cysK</td>
<td>0.29</td>
<td>0.02</td>
<td>0.51</td>
<td>0.08</td>
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<tr>
<td>diaminopimelate decarboxylase</td>
<td>pda</td>
<td>0.29</td>
<td>0.03</td>
<td>n.d.</td>
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<td>#</td>
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<td>30S ribosomal protein S7</td>
<td>rpsG</td>
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<td>0.01</td>
<td>0.98</td>
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<td>rpsT</td>
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<td>#</td>
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<td>peptidoglycan-associated lipoprotein</td>
<td>pal</td>
<td>0.31</td>
<td>0.06</td>
<td>0.74</td>
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<td>GTP cyclohydrolase 1</td>
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<td>0.02</td>
<td>n.d.</td>
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<td>rplF</td>
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<tr>
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<td>0.92</td>
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†, ratio of the amounts of proteins newly synthesized for 10 minutes upon the onset of anaerobiosis and under aerobic conditions determined by quantitation of azhal-containing peptides; ‡, ratio of the total protein levels (pre-existing plus newly synthesized) 10 minutes after the onset of anaerobiosis and under aerobic conditions determined by quantitation of peptides lacking both azhal and methionine; n.d. ribosomal proteins, n.d. not determined.
Changes in newly synthesized proteins are predominantly the result of an altered translation rate rather than an altered degradation-rate—Changes in levels of newly synthesized proteins after a pulse of 10 minutes could be the result of a change in synthesis-rate, degradation-rate or both. The vast majority of proteins in growing *E. coli* cells is relatively stable (109) with half-lives that exceed 2h (110, 111). Proteins of the small pool that is rapidly degraded often have regulatory functions (90). However, no proteome-wide information on protein turnover for each individual protein is available.

To identify proteins of which the change in expression is predominantly the result of a change in translation rate rather than a change in degradation-rate we also analyzed the ‘un-shifted’ fractions obtained during COFRADIC by LC tandem-MS. Peptides in these fractions that do not contain azhal or methionine can be used to quantify changes in total protein levels during the pulse, since they originate from both pre-existing and newly synthesized proteins. In addition, shifted reversed phase HPLC fractions, enriched for azhal-containing peptides, also contain peptides without azhal after secondary runs of COFRADIC. Their presence is due to tailing of main chromatographic peaks that contain the bulk of unlabeled non-shifting peptides. The combined database searches from data acquired from shifted and non-shifted pooled fractions yielded a total of 1451 peptides not containing azhal or methionine, identifying 344 proteins. Of these a total of 305 proteins could be quantified using the reporter ions from iTRAQ. Ten minutes after a switch to an anaerobic environment the total relative amounts of 6 proteins had changed significantly (p<0.05) by a factor of two or more, while 12 proteins changed 1.5-2 fold (p<0.05) and 287 proteins changed less than 1.5 fold or had not changed significantly. Increases in total protein levels within ten minutes after the switch were detected for enzymes involved in fermentation, anaerobic respiration, glycolysis, and PTS (Supplemental Figure 1).

Our datasets of azhal/methionine-lacking and azhal-containing peptides contained 103 proteins of which both the relative total levels during the pulse of 10 minutes and the relative levels of newly synthesized species had been determined. The two datasets are combined in tables I and II and in Figure 2. In Figure 2 the data are ordered according to the relative levels of newly synthesized proteins (dots), while corresponding total levels are represented by diamonds at the same position along the X-axis. As one would expect, the relative total levels (diamonds) of the great majority of proteins change much less than the corresponding relative levels of newly synthesized species (dots). However, we also identified six proteins, GrcA, ClpA, KatG, AhpC, MetN and AhpF, of which the relative total levels changed significantly upon anaerobiosis, almost as much as the relative levels of the newly synthesized species (Figure 2a). Apparently, practically all pre-existing protein molecules of this group have been replaced by newly synthesized polypeptides during the pulse. So, the half-life of these proteins lies well within the pulse-labelling time used. The high turnover of GrcA (115) and ClpA (264, 265) have been found before, while catalase (KatG) has been identified as a putative substrate for the protease ClpB (100). Interestingly AhpC, subunit of a peroxidase, is also identified as a labile protein in a dataset of relative levels of...
newly synthesized and total protein after a change in growth temperature (Chapter 4) under conditions as described before (141), which confirms the observations during the switch to anaerobiosis. Changes in KatG and AhpF are small under these conditions of heat shock. However, changes in protein synthesis of AhpF still seem to be mirrored by changes in total protein levels on the same time-scale. Among the other proteins identified to be labile proteins following heat shock, are the heat shock sigma factor σ^32 (Rps32) and IbpB, a member of the family of small heat shock proteins, both known to have a high turnover rate (Chapter 4), further confirming that the observations relate to turnover rates of the proteins measured. For these labile proteins the change in the level of newly synthesized species could be caused by a change in synthesis-rate, degradation-rate or a combination of the two.

In contrast to the proteins for which the relative total levels change to almost the same degree as the relative newly synthesized levels, a significant difference between relative total levels and relative levels of newly synthesized material after a pulse of 10 minutes was noted for the majority of proteins. This is shown in Figure 2b by red symbols for mixed acid fermentation and anaerobic respiration enzymes, by green symbols for the glycolytic enzymes and proteins of the PTS system, and by blue symbols for ribosomal proteins. For the ribosomal proteins the same trend was again observed during a change in growth temperature (Chapter 4). The difference between levels of total and newly synthesized protein can be explained by assuming that these proteins have a half-life that (far) exceeds the pulse-labelling time used. Consequently, a large change in synthesis will not affect the total protein levels to the same extent in the short time frame used for pulse-labelling; only a continued altered rate of formation will eventually change the protein levels to the same extent. For these stable proteins the effect of degradation-rate is negligible in the pulse-labelling time used and the relative changes in newly synthesized species directly reflect the relative translation rates of these proteins during the pulse of cells grown in aerobic or anaerobic conditions. An example is the 13.5 fold increase of average synthesis-rate found for PibB during 10 minutes after the switch to anaerobiosis by Smith et al. (124) using radiolabelling, which is in good agreement with the 7.8 fold upregulation of the synthesis-rate found with azhal pulse-labelling.

A model for changes in newly synthesized protein levels related to changes in total protein levels during the pulse is presented in the supplemental data. It is clear from the example using glycolytic enzymes (Supplemental Table I) that the measured changes in total levels and in protein synthesis-rate is best approximated by assuming a low turnover (half-life ~2h) for these proteins. This indicates that the increased level of newly synthesized glycolytic enzymes during the pulse is predominantly the result of an increased rate of protein synthesis rather than a decrease in the rate of degradation. In general, the relative amounts of most newly synthesized proteins reflect the average relative translation rates during the pulse.

**DISCUSSION**

This study demonstrates how the proteome-wide azhal pulse-labelling technique combined with COFRADIC enrichment can be used to obtain reliable quantitative data regarding newly synthesized proteins during initial phases of environmental transitions. Although the approach thus far has been based on the subset of methionine containing peptides to identify and quantify newly synthesized proteins, no bias towards proteins with higher methionine content was found compared to the standard approach when all tryptic peptides are employed. Extension of the approach to also determine changes in total protein levels on the same time-scale as new protein synthesis, allows identification of stable and labile proteins. Turnover was determined to be low for most proteins that exhibited a sharp change in new protein formation upon anaerobiosis. This is consistent with earlier notions on protein turnover in *E. coli* (90, 109-111) and correlates well with previous reports on protein turnover in other organisms (116, 119). On the whole this indicates that the change in levels of newly synthesized, stable proteins is caused by a change in protein translation rate, as degradation does not contribute significantly to changes in levels of new protein formation, on the time-scale employed.

The early response of *E. coli* to an anaerobic environment was found to consist of an immediate and strong increase in synthesis-rate of proteins involved in anaerobic respiration and fermentation. Furthermore the synthesis-rates of glycolytic enzymes and PTS proteins increased as well. This strongly suggests that the increased flux through glycolysis (255) is not exclusively the result of metabolic regulation but is also regulated at the protein level. Most of the proteins found to be down-regulated in synthesis were ribosomal proteins. Their rate of synthesis is strongly related to growth rate (262, 263). It has been well documented that under conditions of energy deficiency synthesis of rRNA is decreased (266), leading to translational feedback inhibition of ribosomal protein synthesis (53). The early changes in synthesis-rate upon the anaerobic switch for some of the proteins measured seem to be a good early indication of the steady state levels measured under anaerobic growth, as can be derived from the study of Smith et al. (124) for enolase, pyruvate kinase I, pyruvate-formate lyase B and glyceraldehyde 3-phosphate dehydrogenase. Consequently, measuring changes in the rate of synthesis seems to be a more sensitive indicator of regulation on a short time-scale than determination of changes in protein levels, especially for stable abundant proteins, as is shown by the small changes found in protein levels for these proteins during the first ten minutes after the anaerobic switch.

In addition, using this approach, a number of proteins that have a rapid turnover were also identified. It is unclear whether altered levels of proteins with a short half-life is regulated on the side of the translation or degradation-rate. Of the labile proteins GrcA was found in an earlier study on protein turnover (115), while the half-life of ClpA is also short (264, 265). Upon heat shock, σ^32 and IbpB were identified as labile proteins, in agreement with other studies (106, 107, 242). While KatG was identified as a labile protein after an anaerobic switch, the functionally related proteins of the AhpC/AhpF complex behaved as proteins with...
a short half-life both upon anaerobiosis and heat shock. The functional significance and the mechanisms underlying the rapid turnover of these proteins deserve further investigation.

The increased synthesis-rates of proteins involved in the metabolic adaptations to lack of O_{2} raise questions regarding the relative contribution of regulation at the transcription and/or translational level. Recently, relative mRNA levels have been determined at different time points after a switch to a low oxygen environment in a glucose-limited chemostat-culture of *E. coli* using microarrays (267). Comparison of our protein data with these mRNA data (Supplemental Figure 2) clearly shows discrepancies between relative synthesis-rates and changes in mRNA levels for many proteins including those belonging to the glycolysis, PTS and ribosomal proteins, which suggests regulation at the level of translation rather than transcription. It should be stressed that care should be taken when comparing these datasets due to the difference in culture conditions used, and the candidate proteins suggested here to be regulated at the level of translation should be studied in more detail. Clearly, the anaerobic switch seems an interesting experimental system to further study the extent of transcriptional and translational regulation in *E. coli*.

The current study greatly expands prior knowledge of early changes in protein synthesis after a switch to an anaerobic environment. Furthermore, it allows identification of rapidly and slowly degraded proteins on a proteome-wide scale. In combination with genome-wide data on transcript levels our method to determine relative translation rates of rapidly and slowly degraded proteins on a proteome-wide scale will provide a powerful tool to assess the separate contributions of transcription and translation to the regulation of gene expression.

**Supplemental Data**—Supplemental Figures and Tables can be found in the addendum section on pages 138-144. Protein identification and quantitation data can be found online at [http://www.mcpomline.org](http://www.mcpomline.org) as supplemental information for ref. (141) and (169).

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### Experimental Procedures

**Synthesis of L-azhal**—L-azhal was synthesized from L-Boc-2,4-di-aminobutyric acid (L-Boc-DAB, Chem-Impex, Wood Dale, USA) as described previously (141).

**Cell culture**—The methionine-auxotrophic *E. coli* strain MTD123 (180) was grown in M9 minimal medium as described before (141). For aerobic to anaerobic-switch experiments two cultures (A and B) were grown aerobically at 37°C in M9 minimal medium, in order to have a biological replicate. After overnight culture, cells were inoculated at OD_{600} 0.01 and allowed to grow into exponential phase before being harvested at OD_{600} 1.0. Cells were washed at room temperature with complete M9 minimal medium, but lacking methionine (9). Cells were then split and transferred to either a fully anaerobic culture vessel (under nitrogen) or an aerobic culture vessel both containing M9 minimal medium in which the methionine was replaced by 400 mg/l azhal. The four cultures were allowed to resume growth aerobically or anaerobically for 10 minutes before being harvested.

**Sample preparation and iTRAQ labelling**—Samples were essentially prepared as described before (141), in short: azhal-labelled cells were harvested by centrifugation, pellets were resuspended in lysis buffer and lysed by sonication after which cellular debris was removed by centrifugation. Samples were dialyzed overnight and their protein content was determined. Samples were then subjected to overnight digestion with trypsin, and, for quantitation, digests were labelled with iTRAQ (Applied Biosystems, Toronto, Canada). The digests from the cultures A and B grown under aerobic conditions during pulse-labelling were labelled with iTRAQ 114 and 116 respectively, while digests from the cultures A and B grown anaerobically were labelled with iTRAQ 115 and 117. The four labelled samples were mixed in a 1:1:1:1 (w/w) ratio, based on the protein content of starting material used for trypsin digestion. Excess iTRAQ reagent was removed with a cation-exchange cartridge. Samples were reduced and alkylated before being subjected to COFRADIC.

**COFRADIC and mass spectrometric analysis**—COFRADIC (213) was applied to enrich azhal-containing peptides, using TCEP to selectively modify target peptides between the primary and secondary chromatographic runs (141). TCEP induces a set of competing reactions in azhal-containing peptides present in primary fractions, i.e., conversion of the azido-group to an amine or hydroxyl group and cleavage of the peptide bond at the C-terminal side of azhal residues (141, 143, 222). The subsequent enrichment is based on a difference in retention-time during the secondary chromatographic runs between TCEP-induced reaction products and the bulk of unmodified peptides that are present in the particular primary fraction subjected to TCEP treatment (141). Three fractions, collected 16 minutes apart in the primary run are pooled and reinjected after TCEP treatment. Shifted fractions of secondary runs enriched in TCEP-induced reaction products from azhal-containing peptides, were analyzed by LC tandem-MS as described in detail previously (141). In addition non-shifted pooled fractions were collected, resuspended in 400 µl of 0.1% TFA with the addition of 150 pmol of human [Glu1]-Fibrinopeptide B (Sigma-Aldrich) for internal calibration. Of these samples, 5 µl was injected and analysed as described for the shifted fractions. Peak lists were generated in Analyst QS 1.1, using the mascot.dill script version 1.6b23, essentially with settings as described on the MASCOT website ([http://www.matrixscience.com/help/instruments_analyst.html](http://www.matrixscience.com/help/instruments_analyst.html)) with the exception of the precursor mass tolerance for grouping, which was set at 1.0 Da. Assessment of the relative quantity of each protein by analysis of tandem-MS spectra is based on the signal intensities of reporter ions derived from the iTRAQ-moieties of the respective peptides.

**Data analysis**—Proteins were identified by database searching with the tandem-MS data using the MASCOT search engine version 2.1 (Matrix Science, London, United Kingdom) with parameters as described before (141). In addition a peak list from non-shifted fractions was searched with the following parameters: cleavage after lysine or arginine unless followed by proline, allowing up to one missed cleavage, fixed carbamidomethyl cysteine modification, iTRAQ (K) modification. Variable modification used was the iTRAQ (N-terminal) modification. Peptide mass and MS/MS tolerance was set at 0.1 Da. The significance threshold was set to 0.01 resulting in a threshold score of 28. Multidimensional protein identification technology scoring and “require bold red” were applied with an ion score cut-off of 35 to have all peptide matches identified at a p value of >0.01. MASCOT performed fragment ion searches with the above settings in a local database of the *E. coli* K12 proteome (4328 proteins, 1,381,420 residues, release.
11, June 12, 2007, Uniprot consortium). To estimate false positive rates in protein identification we also performed fragment ion searches against a decoy database, which was a shuffled version of the *E. coli* K12 proteome made using the Peakhardt decoy database builder (Medizinisches Proteom Center, Bochum, Germany). False positive identification rates were found to be less than 3.7 percent. The resulting MASCOT data-files of these searches were imported into Quant (245), relative ratios of newly formed proteins were determined by quantitation using the iTRAQ reporter ions of only azhal-containing peptides unique to each protein and stringent criteria for quantitation as described previously (141), while the iTRAQ reporter ions from peptides not containing azhal or methionine were used to quantify changes in protein levels. To ascertain if up- or down-regulation was significant, a double sided Welch’s T-test for each protein was performed, using the Welch-Satterthwaite equation (246) to determine the degrees of freedom, to see whether the mean protein ratio differed significantly from the mean ratio obtained for unregulated proteins ($\mu = 1.04, \sigma = 0.21, n=164$ for newly synthesized proteins and $\mu = 1.04, \sigma = 0.11, n=302$ for protein levels; from ratio of 116/114 and 117/115 reporter ions). Proteins which changed more than 1.5 fold and for which the p-value was adjusted for a false discovery rate of less than 0.05 due to multiple testing (247) were considered to have a significantly altered expression level.