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Proteome-wide Alterations in *Escherichia coli* Translation Rates upon Anaerobiosis

Gertjan Kramer‡§, Richard R. Sprenger§¶, Merel A. Nessen‡‖, Winfried Roseboom‡, Dave Speijer§, Luitzen de Jong‡, M. Joost Teixeira de Mattos**, JaapWillem Back‡ ‡‡, and Chris G. de Koster‡§§

Enzyme reprofiling in bacteria during adaptation from one environmental condition to another may be regulated by both transcription and translation. However, little is known about the contribution of translational regulation. Recently, we have developed a pulse labeling method using the methionine analog azidohomoalanine to determine the relative amounts of proteins synthesized by *Escherichia coli* in a brief time frame upon a change in environmental conditions. Here we present an extension of our analytical strategy, which entails measuring changes in total protein levels on the same time scale as new protein synthesis. This allows identification of stable and labile proteins and demonstrates that altered levels of most newly synthesized proteins are the result of a change in translation rate rather than degradation rate. With this extended strategy, average relative translation rates for 10 min immediately after a switch from aerobiosis to anaerobiosis were determined. 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 be used to compare relative translation rates with relative mRNA levels at the same time. Discrepancies between these parameters may reveal genes whose expression is regulated by translation rather than by transcription. This may help unravel molecular mechanism underlying changes in translation rates, *e.g.* mediated by small regulatory RNAs. *Molecular & Cellular Proteomics 9:2508–2516, 2010.*

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 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)[1] in *Escherichia coli* and other bacteria (1–4). These sRNAs may regulate translation of numerous mRNAs (5) often mediated by the RNA chaperone Hfq (6). Although the function of many sRNAs is not yet known, several recently reviewed findings (7) 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 (8). Identifying potential target genes is an important step in unraveling underlying molecular mechanisms of translational regulation.

What is needed is a proteomics 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 labeling technique using the methionine analog azidohomoalanine (azhal). This enables assessment of the relative amounts of proteins synthesized in a brief period during adaptation to a major environmental change on a proteomic scale (9). We previously applied this approach to examine early changes in newly synthesized proteins upon a

From ‡Mass Spectrometry of Biomacromolecules and **Molecular Microbial Physiology, Swammerdam Institute for Life Sciences and ‖Biomolecular Synthesis, Van 't Hoff Institute for Molecular Sciences, Universiteit van Amsterdam, Kruislaan 904, 1098 XH Amsterdam, The Netherlands and §Department of Medical Biochemistry, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

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[1] The abbreviations used are: sRNA, small regulatory RNA; azhal, azidohomoalanine; TCEP, tris(2-carboxyethyl)phosphine; COFRADIC, combined fractional diagonal chromatography; Boc, *t*-butoxycarbonyl; iTRAQ, isobaric tags for relative and absolute quantitation; PTS, phosphotransferase system; PFL, pyruvate formate-lyase.

sudden rise in growth temperature. However, the changed levels of newly synthesized proteins after a pulse of several minutes could be the result of a change in either synthesis rate, degradation rate, or a combination of both. A prerequisite to identify proteins with altered translation rates is that the protein half-life far exceeds the labeling time used. The vast majority of abundant proteins in growing *E. coli* cells is relatively stable (10) and has a half-life of at least a few hours (11, 12), whereas a small pool is rapidly degraded (13). However, no proteome-wide information on protein turnover for individual proteins in *E. coli* is available. Here we show that our analytical strategy to determine relative amounts of newly synthesized proteins by pulse labeling with azhal can be easily extended to identify stable and labile proteins. Because degradation of stable newly synthesized proteins during a 10-min pulse is negligible, increased or decreased newly synthesized amounts of stable proteins are predominantly the result of a change in translation rate rather than in degradation rate.

In this study, we used the extended azhal pulse labeling approach and quantitative mass spectrometry to determine, on a proteomic scale, changes in both newly synthesized proteins and 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 with changes in total protein levels in the initial 10 min following the anaerobic switch, stable and labile proteins were 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 labeling time used. We obtained a data set of relative translation rates that is consistently related to the required metabolic adaptation, underscoring the reliability of our method.

EXPERIMENTAL PROCEDURES

Synthesis of L-Azhal—L-Azhal was synthesized from L-Boc-2,4-diaminobutyric acid (Chem-Impex, Wood Dale, IL) as described previously (9).

Cell Culture—The methionine auxotrophic *E. coli* strain MTD123 (14) was grown in M9 minimal medium as described before (9). For aerobic to anaerobic switch experiments, two cultures (A and B) were grown aerobically at 37 °C in M9 minimal medium to have a biological replicate. After overnight culture, cells were inoculated at A_{600} 0.01 and allowed to grow into exponential phase before being harvested at A_{600} 1.0. Cells were washed at room temperature with complete M9 minimal medium but lacking methionine. 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/liter azhal. The four cultures were allowed to resume growth aerobically or anaerobically for 10 min before being harvested.

Sample Preparation and iTRAQ Labeling—Samples were essentially prepared as described before (9). In short, azhal-labeled cells were harvested by centrifugation, and pellets were resuspended in lysis buffer and lysed by sonication after which cellular debris were 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 labeled with iTRAQ (Applied Biosystems, Toronto, Canada). The digests from cultures A and B grown under aerobic conditions during pulse labeling were labeled with iTRAQ 114 and 116, respectively, whereas digests from cultures A and B grown anaerobically were labeled with iTRAQ 115 and 117, respectively. The four labeled 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 combined fractional diagonal chromatography (COFRADIC).

COFRADIC and Mass Spectrometric Analysis—COFRADIC (15) was applied to enrich azhal-containing peptides using TCEP to selectively modify target peptides between the primary and secondary chromatographic runs (9). 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 (9, 16, 17). 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 (9). Three fractions 16 min 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 liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-tandem MS) as described in detail previously (9). In addition, non-shifted pooled fractions were collected, resuspended in 400 μl of 0.1% TFA with the addition of 150 pmol of human [Glu^1]fibrinopeptide B (Sigma-Aldrich) for internal calibration. Of these samples, 5 μl was injected and analyzed as described for the shifted fractions. Peak lists were generated in Analyst QS 1.1 using mascot.dll script version 1.6b23 essentially with settings as described on the MASCOT web site (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 MASCOT search engine version 2.1 (Matrix Science, London, UK) with parameters as described before (9). 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 carbamidomethylcysteine modification, and iTRAQ (Lys) modification. The 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 cutoff 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, Uni-Prot 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%. The resulting MASCOT data files of these searches were imported into Quant (18), and 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 (9), whereas the iTRAQ reporter ions from peptides not containing azhal or methionine were used to quantify changes in protein levels. To ascertain whether up- or down-regulation was significant, a double sided Welch's *t* test for each protein was performed using the Welch-Satterthwaite equation (19) 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 the ratio of 116/114 and 117/115 reporter ions). Proteins that changed more than 1.5-fold and for which the *p* value was adjusted for a false discovery rate of less then 0.05 due to multiple testing (20) were considered to have a significantly altered expression level.

RESULTS

Growth, Labeling, and Quantitation of Newly Synthesized Proteins upon Anaerobic Switch—E. coli is a facultative anaerobe prokaryote, and its ability to switch between aerobic and anaerobic environments greatly expands the range of niches in which it can thrive. 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 because molecular oxygen, the terminal electron acceptor of the aerobic electron transport chain, is no longer available to remove reducing equivalents formed in catabolism. This can be achieved by anaerobic respiration using one or more alternative terminal electron acceptors (21). If no alternative electron acceptor is available, growth on glucose requires removal of reducing equivalents by mixed acid fermentation (22). When grown in aerobic batch cultures with glucose as the energy source and in the absence of alternative electron acceptors, *E. coli* stops growing upon a switch to anaerobiosis for about 20 min and then resumes growth at a slower pace (23). Under these conditions, the flux through the phosphotransferase system (PTS) for glucose transport annex phosphorylation and through glycolysis is dramatically increased (24). 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, transferred to either an aerobic or anaerobic environment, and pulse-labeled for 10 min with azhal. During the pulse of azhal, aerobically cultured cells continued to grow and increased their cellular mass by ~6% in 10 min, judged from an increase in optical density at 600 nm, whereas the anaerobic cells stopped growing in agreement with earlier observations (23).

Proteins extracted from azhal-labeled cultures were digested, and peptides were labeled with iTRAQ for quantitation. Subsequently, azhal-containing peptides were enriched by COFRADIC and identified and quantified by LC-tandem MS as outlined under "Experimental Procedures" and described previously (9). In total, 414 azhal-containing peptides were identified, corresponding to 211 different proteins synthesized during the pulse labeling period (supplemental Table I). Because only methionine-containing peptides can be labeled with azhal and used for the identification of newly synthesized proteins, the number of available peptides per protein that can be used for identification and quantitation sharply declines as shown in the supplemental data using an *in silico* digest (supplemental Fig. 1). Consequently, the contribution of single peptide protein identifications increases as compared with the standard approach when all tryptic peptides can be used for identification (supplemental Table II). However, a large part of the *E. coli* proteome (93.1%) is still predicted to be represented by this subset of peptides (supplemental Fig. 1), and proteins identified do not seem to be biased toward higher methionine content (supplemental Table II).

From the 211 newly synthesized proteins, 164 could be quantified using the iTRAQ reporter ions according to the criteria mentioned under "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 2-fold. 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 min after the anoxic switch (Fig. 1 and supplemental Table III).

Pathways Affected in Rate of Protein Synthesis by Sudden Change to Anaerobic Environment—The change to an anaerobic environment necessitates activation of either the anaerobic respiration machinery or fermentation pathways. Five alternative terminal reductases can be expressed in *E. coli* (21). We detected newly synthesized subunits from four of these, nitrite reductase (NirB), DMSO reductase (DmsA), nitrate reductase (NarG), and fumarate reductase (FrdA); all were increased at least 5-fold. It is noteworthy that the synthesis of NirB, DmsA, and NarG rapidly increases, although 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 among which are the anaerobic respiratory enzymes trimethylamine *N*-oxide reductase, nitrate reductase, and DMSO reductase (25). Furthermore, we found increased amounts of newly synthesized cytochrome-*d* oxidase (CydA) and a putative quinone oxidoreductase (YhdH) (26). The cytochrome-*d* oxidase is known to be expressed under conditions with low oxygen tensions (27), whereas 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 (Fig. 1*a*, *black circles*, and Table I). Pyruvate formate-lyase (PFL), to-

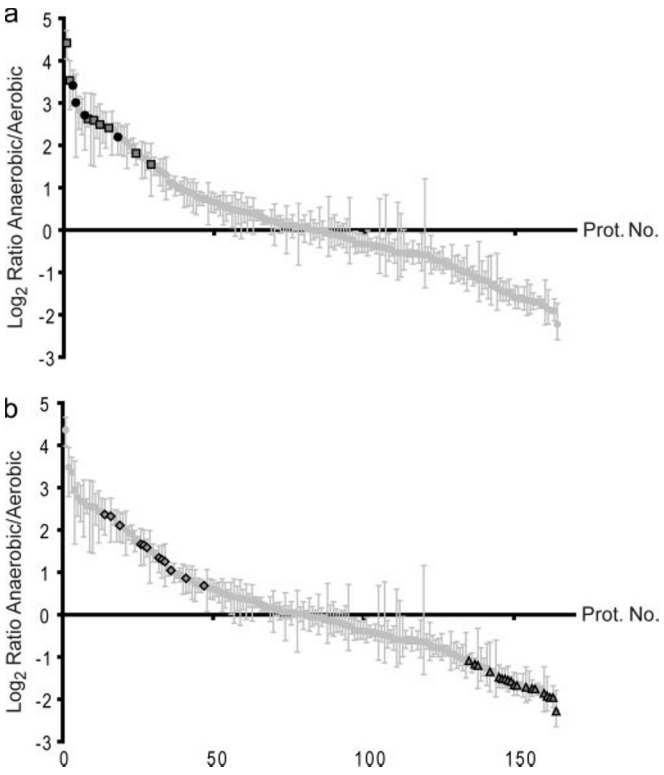

FIG. 1. **Relative levels of proteins newly synthesized during pulse labeling with azhal.** *Light gray circles*, $\log_2$ ratio of newly formed proteins ordered from most increased to most decreased under anaerobic conditions. *Error bars* indicate S.D. Proteins belonging to different pathways are listed in Tables I and II. *a*, *dark gray squares*, proteins involved in anaerobic respiration; *black circles*, mixed acid fermentation enzymes. *b*, *gray diamonds*, glycolytic enzymes and PTS proteins; *dark gray triangles*, ribosomal proteins. *Prot.*, protein.

gether with its auxiliary glycyl radical cofactor (GrcA) (28), plays a central role in this respect as it supplants pyruvate dehydrogenase 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 were also strongly induced upon the anaerobic switch, namely alcohol dehydrogenase (AdhE), which catalyzes the conversion of acetyl-coA to ethanol, and D-lactate dehydrogenase (LdhA), which reduces pyruvate to lactate. Not all proteins involved in fermentation showed dramatic up-regulation in synthesis during the first 10 min upon anaerobic switching. AckA, involved in the conversion of acetyl-coA to acetate, was only modestly up-regulated during the initial response to an anaerobic environment. The relative levels of four newly synthesized enzymes (PflB, GrcA, AdhE, and LdhA) of the six enzymes involved in the conversion of pyruvate to formate, acetate, ethanol, and lactate were increased at least 4-fold, whereas one (AckA) was increased slightly, and the other one (Pta) was not observed. It is also noteworthy that levels of the newly synthesized pyruvate dehydrogenase complex subunits AceE, AceF, and LpdA hardly changed upon anaerobiosis even though pyruvate dehydrogenase activity is strongly inhibited under anaerobic conditions (24).

Another major pathway that was affected by a switch to anaerobic growth in the absence of alternative electron acceptors is glycolysis. It is the pathway that is responsible for the generation of most energy from glucose under these conditions. Fig. 1*b* (*gray diamonds*) shows that the relative amounts of the nine detected glycolytic enzymes synthesized during the first 10 min after the anaerobic switch increased between 1.6- and 5.2-fold (Table I). This represents ~90% of the entire pathway; the only glycolytic enzyme not observed was triose-phosphate isomerase. Our results corroborate and greatly extend previous observations regarding increases in levels of newly synthesized glycolytic and mixed acid fermentation enzymes (23).

In addition, we observed 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 (Fig. 1*b*, *gray diamonds*, and Table I). The PTS protein HPr is lacking in our data set 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 also did not detect one other PTS protein, PtsG, a membrane protein responsible for transport and concomitant phosphorylation of glucose. Despite the lack of some proteins in our data set, these results indicate that the relative amount of most if not all newly synthesized members of the entire PTS and glycolysis increased considerably 10 min after the onset of anaerobiosis.

The synthesis of three uniform stress proteins (29), *i.e.* UspD, UspA, and UspG, was found to increase about 6-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 (30), and their up-regulation 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 decrease under these conditions. Indeed, the levels of all 20 detected newly synthesized ribosomal proteins (of a total of 56), the expression of which is strongly related to growth rate (31, 32), were significantly lower in the anaerobic cells (Fig. 1*b*, *dark gray triangles*, and Table II). Because of their abundance, the synthesis rates of ribosomal proteins contribute considerably to the overall protein synthesis rate. Overall, both the

TABLE I
Identity of newly synthesized proteins most elevated 10 min after switch to anaerobiosis in E. coli

Protein name	Gene	Ratio[a]	S.D.	Ratio[b]	S.D.	Pathway
Nitrite reductase (NAD(P)H) large subunit	*nirB*	20.6	4.7	ND[c]		[d]
Anaerobic dimethyl sulfoxide reductase	*dmsA*	11.2	4.2	ND		[d]
Aldehyde-alcohol dehydrogenase	*adhE*	10.3	2.9	2.6	0.68	[e]
Pyruvate formate-lyase	*pflB*	7.8	4.6	2.3	0.73	[e]
Universal stress protein D	*yiiT*	6.8	1.8	ND		
Universal stress protein A	*uspA*	6.4	1.9	1.5	0.17	
Autonomous glycyl radical cofactor	*grcA*	6.3	2.7	5.4	1.6	[e]
Glycerol dehydrogenase	*gldA*	5.9	0.72	ND		
Universal stress protein G	*ybdQ*	5.9	3.1	ND		
Respiratory nitrate reductase 1	*narG*	5.8	3.1	ND		[d]
Ketol-acid reductoisomerase	*ilvC*	5.8	1.5	ND		
Fumarate reductase flavoprotein subunit	*frdA*	5.4	2.2	ND		[d]
Ribosome-associated inhibitor A	*raiA*	5.4	1.3	ND		
6-Phosphofructokinase isozyme 1	*pfkA*	5.2	1.5	1.8	0.27	[f]
Putative quinone oxidoreductase YhdH	*yhdH*	5.1	0.27	ND		[d]
Fructose-specific phosphotransferase IIA	*fruB*	5.0	1.7	ND		[f]
7-α-Hydroxysteroid dehydrogenase	*hdhA*	5.0	0.61	0.95	0.07	
D-Lactate dehydrogenase	*ldhA*	4.4	1.1	ND		[e]
Phosphoglyceromutase	*gpmI*	4.3	1.0	1.4	0.10	[f]
Bacterioferritin	*bfr*	4.2	1.0	ND		
Probable σ (54) modulation protein	*yhbH*	4.0	1.4	ND		
Malate synthase G	*glcB*	3.8	0.42	ND		
Molybdenum cofactor biosynthesis protein B	*moaB*	3.8	0.57	ND		
Cytochrome-*d* ubiquinol oxidase subunit 1	*cydA*	3.4	0.59	1.7	0.24	[d]
Small heat shock protein IbpB	*ibpB*	3.3	0.26	ND		
Phosphoglycerate kinase	*pgk*	3.2	0.94	1.3	0.21	[f]
Pyruvate kinase II	*pykA*	3.1	0.87	1.2	0.16	[f]
Glucose-6-phosphate isomerase	*pgi*	3.0	0.43	1.2	0.38	[f]
Molybdopterin-converting factor subunit 2	*moaE*	2.8	0.41	ND		
UPF0265 protein YeeX	*yeeX*	2.6	0.25	1.4	0.30	
Phosphoenolpyruvate-protein phosphotransferase	*ptsI*	2.5	0.55	1.1	0.13	[f]
Glyceraldehyde-3-phosphate dehydrogenase A	*gapA*	2.5	0.64	1.2	0.16	[f]
Enolase	*eno*	2.4	0.80	1.1	0.21	[f]
6-Phosphogluconolactonase	*pgl*	2.1	0.24	ND		
Fructose-bisphosphate aldolase class 2	*fbaA*	2.1	0.14	1.1	0.13	[f]
Threonyl-tRNA synthetase	*thrS*	2.0	0.32	1.1	0.18	

[a] Ratio of the amounts of proteins newly synthesized for 10 min upon the onset of anaerobiosis and under aerobic conditions determined by quantitation of azhal-containing peptides.

[b] Ratio of the total protein levels (pre-existing plus newly synthesized) 10 min after the onset of anaerobiosis and under aerobic conditions determined by quantitation of peptides lacking both azhal and methionine.

[c] ND, not determined.

[d] Proteins involved in anaerobic respiration.

[e] Proteins involved in fermentation.

[f] Glycolytic enzymes and PTS sugar transport proteins.

proteins found to be up-regulated and 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 data set.

Changes in Newly Synthesized Proteins Are Predominantly the Result of Altered Translation Rate Rather than Altered Degradation Rate—Changes in levels of newly synthesized proteins after a pulse of 10 min 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 (10) with half-lives that exceed 2 h (11, 12). Proteins of the small pool that is rapidly degraded often have regulatory functions (13). 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 "unshifted" 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 because they originate from both pre-existing and newly synthesized proteins. In addition, shifted reversed phase HPLC fractions, enriched for azhal-containing peptides, also contain

TABLE II
Identity of newly synthesized proteins most decreased 10 min after switch to anaerobiosis in E. coli

Protein name	Gene	Ratio[a]	S.D.	Ratio[b]	S.D.	Pathway
50 S ribosomal protein L11	*rplK*	0.21	0.06	0.95	0.27	[c]
50 S ribosomal protein L4	*rplD*	0.26	0.05	0.86	0.13	[c]
30 S ribosomal protein S8	*rpsH*	0.26	0.01	0.91	0.07	[c]
30 S ribosomal protein S10	*rpsJ*	0.26	0.07	0.92	0.17	[c]
50 S ribosomal protein L2	*rplB*	0.28	0.10	0.86	0.13	[c]
Cysteine synthase A	*cysK*	0.29	0.02	0.51	0.08	
Diaminopimelate decarboxylase	*lysA*	0.29	0.03	ND[d]		
30 S ribosomal protein S7	*rpsG*	0.30	0.01	0.98	0.17	[c]
30 S ribosomal protein S20	*rpsT*	0.30	0.04	0.83	0.17	[c]
Peptide deformylase	*def*	0.30	0.07	ND		
50 S ribosomal protein L1	*rplA*	0.31	0.09	0.89	0.15	[c]
Peptidoglycan-associated lipoprotein	*pal*	0.31	0.06	0.74	0.13	
GTP cyclohydrolase 1	*folE*	0.31	0.02	ND		
50 S ribosomal protein L6	*rplF*	0.31	0.06	0.94	0.08	[c]
50 S ribosomal protein L13	*rplM*	0.32	0.06	0.95	0.11	[c]
50 S ribosomal protein L9	*rplI*	0.34	0.03	0.93	0.16	[c]
30 S ribosomal protein S9	*rpsI*	0.34	0.07	0.92	0.18	[c]
30 S ribosomal protein S1	*rpsA*	0.35	0.05	1.02	0.20	[c]
50 S ribosomal protein L3	*rplC*	0.35	0.06	0.85	0.15	[c]
50 S ribosomal protein L17	*rplQ*	0.36	0.07	0.83	0.11	[c]
Alkyl hydroperoxide reductase subunit F	*ahpF*	0.37	0.16	0.49	0.14	
Quinoprotein glucose dehydrogenase	*gcd*	0.39	0.02	ND		
50 S ribosomal protein L5	*rplE*	0.39	0.15	0.90	0.13	[c]
Biopolymer transport protein ExbB	*exbB*	0.42	0.14	ND		
β-Hydroxydecanoyl thioester dehydrase	*fabA*	0.42	0.06	0.93	0.10	
Polyribonucleotide nucleotidyltransferase	*pnp*	0.43	0.11	0.75	0.17	
30 S ribosomal protein S5	*rpsE*	0.43	0.20	0.90	0.12	[c]
50 S ribosomal protein L21	*rplU*	0.44	0.04	0.90	0.16	[c]
DNA-directed RNA polymerase subunit β	*rpoB*	0.45	0.07	0.96	0.15	
30 S ribosomal protein S2	*rpsB*	0.47	0.04	0.93	0.13	[c]
Magnesium-transporting ATPase, P-type 1	*mgtA*	0.48	0.17	ND		
Cold shock DEAD box protein A	*deaD*	0.48	0.10	ND		
Uridylate kinase	*pyrH*	0.50	0.07	ND		

[a] Ratio of the amounts of proteins newly synthesized for 10 min upon the onset of anaerobiosis and under aerobic conditions determined by quantitation of azhal-containing peptides.
[b] Ratio of the total protein levels (pre-existing plus newly synthesized) 10 min after the onset of anaerobiosis and under aerobic conditions determined by quantitation of peptides lacking both azhal and methionine.
[c] Ribosomal proteins.
[d] ND, not determined.

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 (supplemental Table IV), identifying 344 proteins. Of these, a total of 305 proteins could be quantified using the reporter ions from iTRAQ (supplemental Table V). Ten minutes after a switch to an anaerobic environment the total relative amounts of six proteins had changed significantly ($p < 0.05$) by a factor of 2 or more, whereas 12 proteins changed 1.5–2-fold ($p < 0.05$), and 287 proteins changed less then 1.5-fold or had not changed significantly. Increases in total protein levels within 10 min after the switch were detected for enzymes involved in fermentation, anaerobic respiration, glycolysis, and PTS (supplemental Fig. 2).

Our data sets of azhal/methionine-lacking and azhal-containing peptides contained 103 proteins of which both the relative total levels during the pulse of 10 min and the relative levels of newly synthesized species had been determined (supplemental Table VI). The two data sets are combined in Tables I and II and in Fig. 2. In Fig. 2, the data are ordered according to the relative levels of newly synthesized proteins (*circles*), whereas 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 changed much less than the corresponding relative levels of newly synthesized species (*circles*). 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 (Fig. 2*a*). Apparently, practically all pre-existing protein molecules of this group

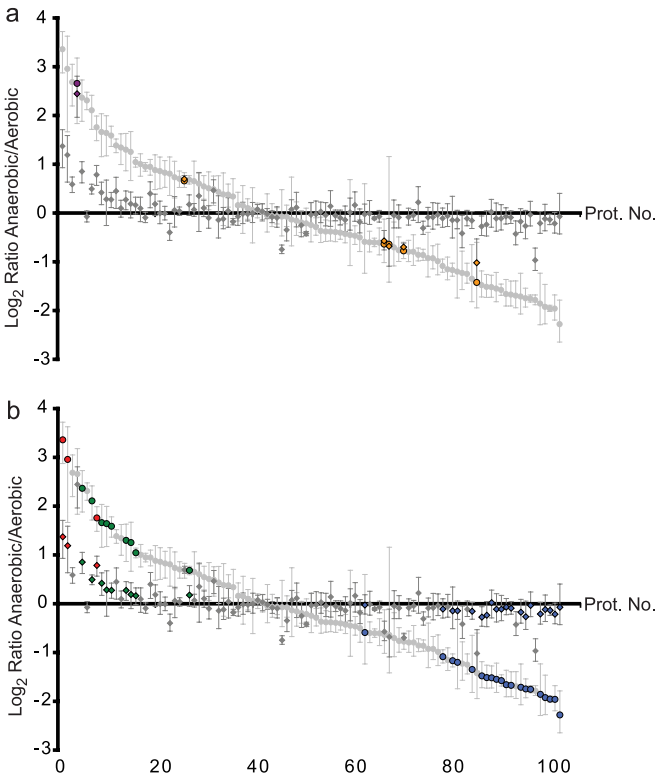

FIG. 2. **Changes in protein synthesis compared with changes in protein levels upon onset of anaerobiosis.** *Light gray* or *colored circles*, relative change of newly synthesized proteins; *dark gray* or *colored diamonds* at the same position on the *x axis* as a *circle* are relative total protein levels of the same protein 10 min after the switch to anaerobiosis. Proteins are ordered from most induced to most repressed synthesis. In *a*, proteins having a *high* turnover rate are marked, namely GrcA (*purple circle* and *diamond*) and ClpA, KatG, AhpC, MetN, and AhpF (*orange circles* and *diamonds*). In *b*, colored symbols are used to represent proteins with a *low* turnover rate in the same data set. *Red circles* and *diamonds*, proteins involved in anaerobic respiration and mixed acid fermentation; *green circles* and *diamonds*, glycolysis; *blue circles* and *diamonds*, ribosomal proteins. *Prot.*, protein.

have been replaced by newly synthesized polypeptides during the pulse. Therefore, the half-life of these proteins lies well within the pulse labeling time used. The high turnover of GrcA (33) and ClpA (34, 35) has been found before, whereas catalase (KatG) has been identified as a putative substrate for the protease ClpB (36). Interestingly, AhpC, a subunit of a peroxidase, was also identified as a labile protein in a data set of relative levels of newly synthesized and total protein after a change in growth temperature (supplemental Fig. 3 and supplemental Tables VII and VIII) under conditions as described before (9), confirming the observations during the aerobic switch. Changes in KatG and AhpF were 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 (supplemental Fig. 3). Among the other proteins identified to be labile proteins following heat shock are the heat shock sigma factor σ^{32} (Rp32) and IbpB, a member of the family of small heat shock proteins, both known to have a high turnover rate (37–39), further confirming that the observations relate to turnover rate 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 changed 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 min was noted for the majority of proteins. This is shown in Fig. 2*b* by *red* symbols for mixed acid fermentation and anaerobic respiration enzymes, by *green* symbols for the glycolytic enzymes and proteins of the PTS, and by *blue* symbols for ribosomal proteins. For the ribosomal proteins, the same trend was again observed during a change in growth temperature (supplemental Fig. 3). 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 labeling time used. Their synthesis and degradation rates are small compared with their total cellular levels under exponential growth. 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 labeling; 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 labeling 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 PflB during 10 min after the switch to anaerobiosis by Smith and Neidhardt (23) using radiolabeling that is in good agreement with the 7.8-fold up-regulation of synthesis found with azhal pulse labeling.

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 IX) that the measured changes in total levels and protein synthesis are best approximated by assuming a low turnover (half-life ~2 h) 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 then 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 labeling technique combined with COFRADIC enrich-

ment 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 toward proteins with higher methionine content was found compared with the standard approach when all tryptic peptides are used. 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* (10–13) and correlates well with previous reports on protein turnover in other organisms (40, 41). 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 used.

The early response of *E. coli* in reaction to an anaerobic environment was found to consist of an immediate and strong increase in the synthesis rates 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 (24) 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 synthesis is strongly related to growth rate (31, 32). It has been well documented that under conditions of energy deficiency synthesis of rRNA is decreased (42), leading to translational feedback inhibition of ribosomal protein synthesis (43). The early changes in synthesis rate upon the anaerobic switch for some of the proteins measured seem to be a good early indication of steady state levels under anaerobic growth as can be derived from the study of Smith and Neidhardt (23) for enolase, pyruvate kinase I, pyruvate-formate lyase B, and glyceraldehyde-3-phosphate dehydrogenase. Consequently, measuring synthesis rate changes seems to be a more sensitive indicator of regulation on a short time scale compared with 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 10 min 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 are regulated on the side of translation or degradation rate. Of the labile proteins, GrcA was found in an earlier study on protein turnover (33), whereas the half-life of ClpA is also short (34, 35). Upon heat shock, σ^{32} and IbpB were identified as labile proteins in agreement with other studies (37–39). Although 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 (44). Comparison of our protein data with these mRNA data (supplemental Fig. 4) clearly shows discrepancies between relative synthesis rates and changes in mRNA levels for many proteins, including those belonging to glycolysis, PTS, and ribosomal proteins, suggesting regulation at the level of translation rather than transcription. It should be stressed that care should be taken when comparing these data sets because of 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 on a proteomic scale will provide a powerful tool to assess the separate contributions of transcription and translation to the regulation of gene expression.

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S This article contains supplemental Tables I–X and Figs. 1–5, annotated tandem MS spectra for single hit peptides, supplemental Materials and Methods and a kinetic model.

¶ Present address: Dept. of Biochemistry and Molecular Biology, University of Southern Demark, Campusvej 55, DK-5230 Odense, Denmark.

‡‡ Present address: Pepscan Therapeutics BV, Zuidersluisweg 2, 8243 RC Lelystad, The Netherlands.

§§ To whom correspondence should be addressed: Mass Spectrometry of Biomacromolecules, Swammerdam Inst. for Life Sciences, Universiteit van Amsterdam, Kruislaan 904, P. O. Box 94215, 1090 GE Amsterdam, The Netherlands. Tel.: 31-205255457; Fax: 31-205257934; E-mail: c.g.dekoster@uva.nl.

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