

# Identification and Quantitation of Newly Synthesized Proteins in *Escherichia coli* by Enrichment of Azidohomoalanine-labeled Peptides with Diagonal Chromatography<sup>S</sup>

Gertjan Kramer‡, Richard R. Sprenger§, Jaap Willem Back‡¶, Henk L. Dekker‡, Merel A. Nessen‡||, Jan H. van Maarseveen||, Leo J. de Koning‡, Klaas J. Hellingwerf\*\*, Luitzen de Jong‡, and Chris G. de Koster‡ ‡‡

A method is presented to identify and quantify several hundreds of newly synthesized proteins in *Escherichia coli* upon pulse labeling cells with the methionine analogue azidohomoalanine (azhal). For the first 30 min after inoculation, a methionine-auxotrophic strain grows equally well on azhal as on methionine. Upon a pulse of 15 min and digestion of total protein, azhal-labeled peptides are isolated by a retention time shift between two reversed phase chromatographic runs. The retention time shift is induced by a reaction selective for the azido group in labeled peptides using tris(2-carboxyethyl)phosphine. Selectively modified peptides are identified by reversed phase liquid chromatography and on-line tandem mass spectrometry. We identified 527 proteins representative of all major Gene Ontology categories. Comparing the relative amounts of 344 proteins synthesized in 15 min upon a switch of growth temperature from 37 to 44 °C showed that nearly 20% increased or decreased more than 2-fold. Among the most up-regulated proteins many were chaperones and proteases in accordance with the cells response to unfolded proteins due to heat stress. Comparison of our data with results from previous microarray experiments revealed the importance of regulation of gene expression at the level of transcription of the most elevated proteins under heat shock conditions and enabled identification of several candidate genes whose expression may predominantly be regulated at the level of translation. This work demonstrates for the first time the use of a bioorthogonal amino acid for proteome-wide detection of changes in the amounts of proteins synthesized

during a brief period upon variations in cellular growth conditions. Comparison of such data with relative mRNA levels enables assessment of the separate contributions of transcription and translation to the regulation of gene expression. *Molecular & Cellular Proteomics* 8: 1599–1611, 2009.

Knowledge about protein synthesis and degradation rates on a proteome-wide scale is an important requirement for advanced modeling of the kinetics of cellular response networks. Pulse-chase labeling with radiolabeled compounds combined with separation of proteins by two-dimensional gel electrophoresis has already been applied (1, 2). However, this approach has drawbacks, such as difficulties to detect very acidic, basic, or hydrophobic proteins (e.g. membrane proteins). The possible occurrence of more than one protein in a gel spot, masking the relative contribution of each species to the total radioactivity, is another intrinsic difficulty.

The use of amino acids labeled with stable isotopes rather than radioisotopes is a solution that is applicable to a mass spectrometry-based proteome-wide approach (3–7). However, this method needs extensive labeling times as the unlabeled bulk of the protein content of the cell will also be detected. Detection of small amounts of labeled, newly synthesized proteins in the presence of large amounts of unlabeled proteins is severely limited by the dynamic range of the mass spectrometer. This requirement for longer labeling times hampers identification and quantitation of transient changes in protein expression following perturbations upon pulse labeling. What is needed is an amino acid analogue that can be distinguished from its natural counterpart, can be used in a gel-free proteomics approach, and will facilitate the isolation of newly synthesized proteins from a large pool of pre-existing proteins. This will enhance identification and increase the dynamic range as well as the sensitivity of detection for transiently expressed proteins.

In recent efforts, non-natural amino acids have been used to distinguish between newly synthesized proteins and pre-

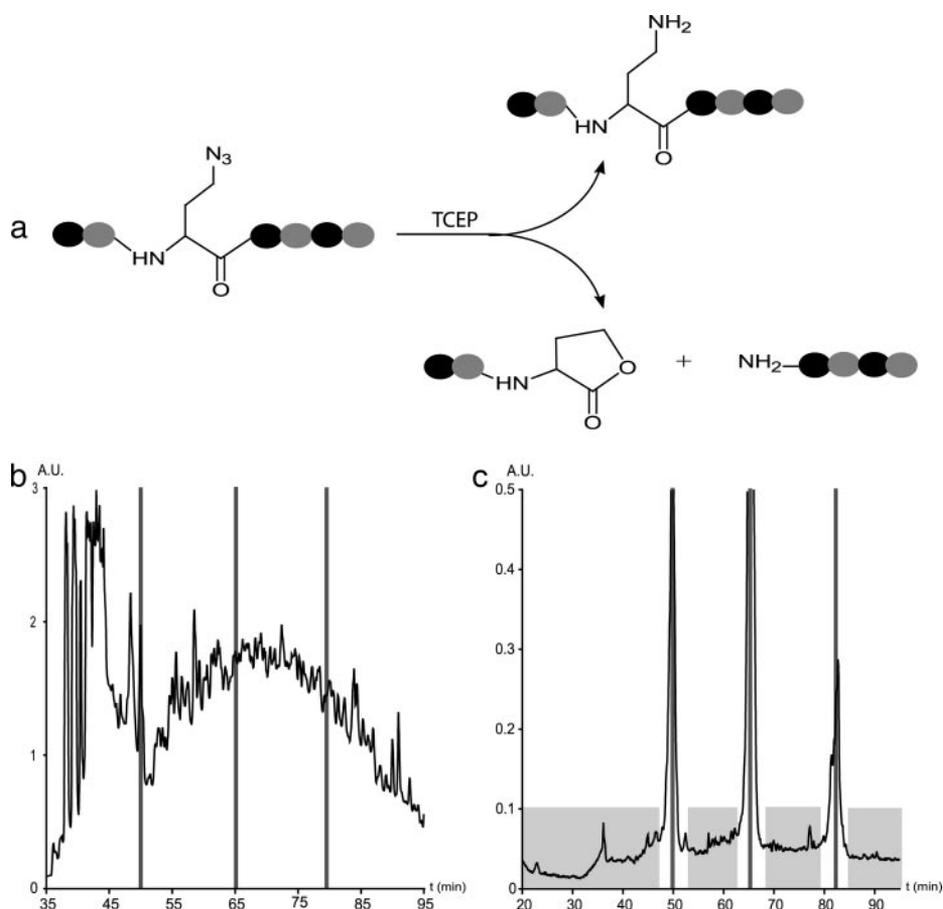
From ‡Mass Spectrometry of Biomacromolecules and \*\*Molecular Microbial Physiology, Swammerdam Institute for Life Sciences, Nieuwe Achtergracht 166, 1018WV Amsterdam, The Netherlands, §Proteomics Group, Department of Medical Biochemistry, Academic Medical Centre, Meibergdreef 15, 1105AZ Amsterdam, The Netherlands, and ||Biomolecular Synthesis, Van't Hoff Institute for Molecular Sciences, Nieuwe Achtergracht 129, 1018WS Amsterdam, The Netherlands

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FIG. 1. TCEP-induced reaction of azhal-labeled peptides and the principle of the COFRADIC approach. *a*,

TCEP treatment of azhal-labeled peptides will induce two competing reaction pathways. In the first, azhal-containing peptides undergo a reaction during which the azide moiety of azhal is converted to an amine and azhal becomes a diaminobutyrate residue. In the second pathway the peptide is cleaved at the azhal residue, yielding an N-terminal cleavage product with a C-terminal homoserine lactone residue and an unmodified C-terminal cleavage product. *b* shows the primary RP HPLC run of a tryptic digest of a labeled *E. coli* proteome with a total of 48 1-min fractions collected. To diminish the number of secondary runs to 16, primary fractions separated by an interval of 16 min were pooled (gray bars). Subsequently pooled fractions were treated with TCEP and run again under identical conditions (*c*). Unlabeled peptides are not modified and will run at the same retention time, whereas labeled peptides either have their azide group reduced to an amine or are cleaved at azhal and shift their retention time. The non-shifted fractions (gray bars) plus three adjacent fractions on the front and back are discarded, whereas fractions containing shifted peptides (gray boxes) are collected, pooled, and analyzed by tandem MS.



existing proteins. Azidohomoalanine (azhal),<sup>1</sup> a methionine analogue, was reported to be efficiently incorporated into recombinant proteins as well as protein complexes produced in methionine-auxotrophic *Escherichia coli* strains and in mammalian cells grown in the presence of the analogue (8–16). Azido groups can be selectively modified, enabling fluorescent labeling of newly synthesized proteins. This for example allows following the movement of newly synthesized proteins through different cellular compartments (17–19). In the bioorthogonal non-canonical amino acid tagging approach (20, 21), cells were grown on a mixture of azhal and deuterated leucine to label newly synthesized proteins. Copper-catalyzed cycloaddition was used to covalently attach a biotin-alkyne affinity handle to those proteins that were labeled with azhal. Biotinylated proteins were then retained on an avidin resin and, after thorough washing, digested directly on the resin to liberate tryptic peptides from the labeled material. Nearly 200 newly synthesized proteins were identified by the detection of peptides that contained either azhal

derivatives or deuterated leucine. Therefore, azhal seems to be a promising label with which to probe protein synthesis and degradation rates on a proteome-wide scale. However, the bioorthogonal non-canonical amino acid tagging approach has not been applied widely yet possibly because the affinity purification procedure includes a few critical steps as explicitly mentioned by the authors (21).

Here we present an alternative to the affinity purification approach for separating azido-labeled peptides from unlabeled peptides. Our approach is based on combined fractional diagonal chromatography (COFRADIC), originally used by Gevaert *et al.* (22) in a proteome-wide approach to identify methionine-containing peptides and also applied to enrich other peptide subpopulations (23–28). Gevaert *et al.* (22) used the oxidation of methionine residues to induce retention time shifts in reversed phase (RP) HPLC to separate methionine-containing from non-methionine-containing peptides. We adapted this approach to isolate azhal-labeled peptides by a retention time shift. The retention time shift is induced by reaction of tris(2-carboxyethyl)phosphine (TCEP) with azhal (14). Two competing reactions are induced in azhal-containing peptides by this reagent. In one pathway the azhal residue is converted into a diaminobutyrate residue via the reduction of the azido group to an amino group (Fig. 1a). The more polar

<sup>1</sup> The abbreviations used are: azhal, azidohomoalanine; TCEP, tris(2-carboxyethyl)phosphine; RP, reversed phase; COFRADIC, combined fractional diagonal chromatography; DAB, diaminobutyrate; iTRAQ, isobaric tags for relative and absolute quantitation; Boc, *t*-butoxycarbonyl; GO, Gene Ontology.

nature of an amino group compared with an azido group induces shifts on RP HPLC. In the other pathway the peptide bond at the C-terminal side of an azhal residue is cleaved. In the N-terminal product of this cleavage reaction the C-terminal azhal has been converted into homoserine lactone; the C-terminal cleavage product is an unmodified peptide (Fig. 1a). Cleavage products will have retention times that differ from the parent compounds to an extent that depends on their change in hydrophobicity. Previously these TCEP-induced reactions were applied in combination with diagonal chromatography to isolate cross-linked peptides with an artificially introduced linker containing an azide moiety (29). Here we demonstrate the ability of this method, *i.e.* azido peptide isolation by COFRADIC, to enrich azhal-labeled peptides and subsequently identify newly synthesized proteins in a proteome-wide approach. We present data on 527 *E. coli* proteins synthesized during a 15-min pulse. Furthermore by using iTRAQ, we show the ability of this method for relative quantitation of changes in the amount of newly synthesized protein after an up-shift in growth temperature from 37 to 44 °C. The early response of changes in protein synthesis upon a change in growth temperature was examined, and quantitative data are presented for 344 proteins. This demonstrates for the first time a proteome-wide, bioorthogonal approach for relative quantitation of proteins synthesized during a small time window upon a change in growth conditions.

#### EXPERIMENTAL PROCEDURES

**Synthesis of L-Azhal**—L-Azhal was synthesized from L-Boc-2,4-diaminobutyric acid (L-Boc-DAB, Chem-Impex) by diazo transfer using triflic azide (TfN<sub>3</sub>) as described previously (30).

**Cell Culture**—The methionine-auxotrophic *E. coli* strain MTD123 (31) was grown aerobically at 37 °C in LB medium. For labeling experiments cells grown overnight in LB medium were transferred to M9 minimal medium containing 6.8 μM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub>, 59.3 μM thiamine-HCl, 57.0 nM Na<sub>2</sub>SeO<sub>3</sub>, 5.0 μM CuCl<sub>2</sub>, 10.0 μM CoCl<sub>2</sub>, 5.2 μM H<sub>3</sub>BO<sub>3</sub>, 99.9 μM FeCl<sub>3</sub>, 50.5 μM MnCl<sub>2</sub>, 25.3 μM ZnO, 0.08 μM Na<sub>4</sub>MoO<sub>4</sub>, 111 mM glucose, and 60 mg/liter for each of the 19 natural amino acids and 40 mg/liter for tyrosine (Sigma-Aldrich). Cells were inoculated at A<sub>600</sub> 0.1 and allowed to grow into exponential phase before being harvested at A<sub>600</sub> 1.0 by centrifugation for 10 min at 4500 rpm and 4 °C. Cells were then washed twice by resuspending the cell pellet in sterile M9 medium without additives followed by centrifugation to eliminate traces of methionine. After washing cells were transferred to M9 minimal medium (see above) in which the methionine was replaced by 400 mg/liter azhal, and cells were allowed to resume growth aerobically at 37 °C. Growth curves were recorded with cells treated as in the labeling experiments except that methionine or azhal conditions were varied as indicated. To determine the number of viable cells, cells were diluted in sterile M9 medium and plated on LB-agar plates in duplicate; colonies were counted after overnight growth at 37 °C. For temperature switch experiments two cultures (A and B) were grown aerobically at 37 °C in M9 minimal medium as described above to have a biological replicate. After overnight culture, cells were inoculated at A<sub>600</sub> 0.01 and allowed to grow into exponential phase before being harvested at A<sub>600</sub> 1.0. These cells were washed at room temperature with complete M9 minimal medium (but lacking methionine) to prevent osmotic shock during washing and then transferred to M9 minimal medium in which

the methionine was replaced by 400 mg/liter azhal. The cultures were split and then transferred to water bath shakers set to either 37 or 44 °C. The four cultures were allowed to resume growth aerobically for 15 min before being harvested.

**Sample Preparation**—Unless stated otherwise, all the following manipulations in the protocol were carried out in “protein low bind tubes” (Eppendorf, Hamburg, Germany) to limit losses due to binding to the tube surface. Azhal-labeled cells were harvested by centrifugation for 10 min at 4500 rpm and 4 °C. Pellets were resuspended in 8 M urea and 50 mM Hepes, pH 8.0 (Sigma-Aldrich), and fragmented by sonication. Lysates were centrifuged for 30 min at 15,000 rpm and 4 °C to remove cellular debris. Next samples were dialyzed against 0.5 M urea and 50 mM Hepes, pH 8.0, overnight at 4 °C or against 10 mM Hepes, pH 8.0, for temperature switch samples. Protein content of dialyzed samples was determined using a bicinchoninic acid-based protein assay kit (32) (Pierce) following the manufacturer’s protocol. Samples were then subjected to overnight digestion at 37 °C using a 1:50 (w/w) protease:protein ratio with trypsin (trypsin gold, mass spectrometry grade, Promega, Madison, WI). Subsequently samples were treated with 2 mM TCEP (BioVectra, Chantlotetown, Canada) for 5 min at room temperature to reduce disulfide bridges. The duration of this TCEP treatment is too short to induce any reactions with azhal but sufficient to reduce the disulfide bridges of the peptides (data not shown). TCEP treatment was followed by incubation with 5 mM sodium azide and 10 mM iodoacetamide (Sigma-Aldrich) in the dark at room temperature for 15 min to oxidize TCEP and alkylate cysteine residues, respectively. For the primary run of diagonal chromatography 500 μg of the resulting protein digest was loaded onto the SMART system.

**iTRAQ Labeling**—For quantitation samples obtained from the temperature switch experiment (125 μg of protein/sample) were lyophilized after digestion. Samples were redissolved in 40 μl of 125 mM triethylammonium bicarbonate, pH 8.5, and labeled with iTRAQ (33) according to the manufacturer’s protocol (Applied Biosystems, Toronto, Canada) with the exception that two vials of iTRAQ reagent were used per sample to ensure complete labeling. Samples were incubated for 2 h at room temperature after which the reaction was quenched by adding 300 μl of 0.1% formic acid. The digests from cultures A and B grown at 37 °C during pulse labeling were labeled with iTRAQ 114 and 116, respectively, whereas digests from cultures A and B grown at 44 °C were labeled with iTRAQ 115 and 117, respectively. The four labeled samples were mixed in a 1:1:1:1 (w/w) ratio, resulting in 500 μg of iTRAQ labeled digest. To remove the excess iTRAQ reagent the sample was diluted three times to a final volume of 6 ml of 20% acetonitrile in 0.1% formic acid and loaded on an ICAT cation exchange cartridge (Applied Biosystems). The cartridge was washed with 500 μl of 20% acetonitrile in 0.1% formic acid before the digest was eluted with 2 M ammonium formate buffer, pH 6.8, containing 20% acetonitrile and lyophilized. Samples were redissolved in 50 mM Hepes, pH 8.0, and reduced and alkylated as described above before ~200 μg was loaded for the primary run of diagonal chromatography as described below.

**Diagonal Chromatography**—Primary and secondary runs of diagonal chromatography for COFRADIC were carried out with a SMART system (Pharmacia, Uppsala, Sweden) equipped with a 200 μl sample loop and a fraction collector, using a Jupiter Proteo C12 column (ID 2 mm, length 150 mm, Phenomenex, Torrance, USA). All solvents used were LC-MS grade (Biosolve, Valkenswaard, The Netherlands). Samples (200 μl) were loaded onto the column using 0.1% trifluoroacetic acid (TFA) in water (Solvent A) at a flow rate of 50 μl/min for 7 min. Then the column was washed with this solvent for another 13 min, before a linear gradient to 50% acetonitrile in 0.1% TFA in 75 min was applied to elute bound peptides. During application of the gradient fractions of 1 min were collected, and the absorbance of the effluent

was continuously monitored at 214, 254, and 280 nm. Fractions of 1 min, collected from 35 to 83 min, were combined into 16 pools, each containing three fractions that were 16 min apart in the primary run (Fig. 1b). These pools were lyophilized overnight and subsequently treated overnight at 40 °C with freshly prepared 10 mM TCEP in 50 mM Hepes, pH 8.0, before being reinjected for the secondary run of diagonal chromatography. Fractions of 1 min were collected in the secondary run from 10 min after the start of the gradient onward. After the secondary run, fractions that corresponded with the original three primary run fractions, judged on the basis of absorbance, plus three adjacent fractions on the front and back of an on-diagonal fraction were discarded (Fig. 1c). The remaining fractions, now termed off-diagonal fractions, which contain the shifted reaction products of peptides initially containing azhal, were pooled and lyophilized overnight before further analysis.

**Mass Spectrometric Analysis**—Off-diagonal pooled fractions were redissolved in 30  $\mu$ l of 0.1% TFA or for the iTRAQ labeled temperature switch sample in 10  $\mu$ l of 0.1% TFA with the addition of 150 pmol of human [Glu<sup>1</sup>]fibrinopeptide B (Sigma-Aldrich) for internal calibration. For tandem MS analyses, 5 or 10  $\mu$ l (temperature switch) of sample was separated using an Agilent 1100 series LC system fitted with a nanoscale RP HPLC setup involving Dean's switching (34) as described by Meiring *et al.* (35). After loading onto a 2-cm  $\times$  100- $\mu$ m-inner diameter C<sub>18</sub> trapping column (Nanoseparations, Biltoven, The Netherlands) and washing for 10 min at a flow rate of 5  $\mu$ l/min with 98% solvent A (0.1% formic acid in water) and 2% solvent B (0.08% formic acid in acetonitrile), the peptides were eluted onto a 63-cm  $\times$  50- $\mu$ m-inner diameter C<sub>18</sub> reversed phase analytical column (Nanoseparations) using a linear gradient of 8–30% solvent B for 95 min at a flow rate of 125 nl/min. The column was interfaced to a QSTAR-XL mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) for on-line ESI-MS via a liquid junction with nebulizer using an uncoated fused silica emitter (New Objective, Cambridge, MA) operating around 4.7 kV (inner diameter, 20  $\mu$ m; tip inner diameter, 10  $\mu$ m). Survey scans were acquired from *m/z* 300 to 1200. The three most intense ions were selected for tandem MS using automatic selection and dynamic exclusion scripts in Analyst QS 1.1 (maximum repetitions; 2; independent data acquisition extensions, II). Peak lists were generated in Analyst QS 1.1 using the mascot.dll script version 1.6b23 essentially with settings as recommended by Matrix Science with the exception of the precursor mass tolerance for grouping, which was set at 1.0 Da.

**Data Analysis**—Generated peak lists were submitted to the MASCOT search engine 2.1 (Matrix Science, London, UK). The MASCOT search parameters were as follows: cleavage after lysine or arginine unless followed by proline plus cleavage after methionine, allowing up to three missed cleavages, fixed carbamidomethylcysteine modification, and carbamylation of lysine and the N terminus as variable modifications. Variable modifications induced by reaction of TCEP with azhal-containing peptides include methionine C-terminally converted to a homoserine lactone after cleaving (analogous to cyanogen bromide cleavage). For the reduction of the azido group a modification was defined on methionine-coded residues as a methionine residue replaced by diaminobutyrate (C<sub>4</sub>H<sub>8</sub>N<sub>2</sub>O; accurate mass, 100.063663 amu) as described previously (14). Besides the reaction products reported earlier, a modification was defined as a methionine residue replaced by homoserine (C<sub>4</sub>H<sub>7</sub>NO<sub>2</sub>; accurate mass, 101.047679 amu). Formation of homoserine from azhal escaped detection in a previous study (14) but was repeatedly observed in the present work. Peptide mass tolerance was set at 0.15 Da, and MS/MS tolerance was set at 0.1 Da. The significance threshold was set to 0.01 resulting in a threshold score of 38. Multidimensional protein identification technology scoring and "require bold red" were applied with an ion score cutoff of 38 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 rates were estimated by dividing the total number of protein hits from the decoy database by the total number of protein hits from the *E. coli* K12 database and multiplying by 100%.

Peptide samples subjected to LC-tandem MS for protein identification with MASCOT contained both TCEP-induced reaction products from azhal-labeled peptides and unmodified peptides not derived from azhal-containing peptides by TCEP treatment. To remove the latter species, we selected manually in MASCOT only those peptides that have the variable modifications homoserine lactone, diaminobutyrate, and homoserine and the unmodified C-terminal peptides that resulted from cleavage after a methionine residue. Then the MASCOT search was performed again with this selection using the same settings as described above to recalculate MASCOT protein scores and protein coverage based solely on reaction products. The resulting proteins, representing newly synthesized proteins made during the labeling time with azhal, were exported as a comma-separated value file for further analysis.

**Quantitation**—The 16 tandem MS runs were first internally recalibrated on the fragmentation spectrum of [Glu<sup>1</sup>]fibrinopeptide B before being exported by the mascot.dll as described above. Generated peak lists were submitted to MASCOT to identify newly synthesized proteins using the following parameters: cleavage after lysine or arginine unless followed by proline plus cleavage after methionine, allowing up to two missed cleavages, fixed carbamidomethyl cysteine, and iTRAQ (Lys) modifications. Variable modifications used were iTRAQ (N-terminal) modification and modifications induced by reaction of TCEP with azhal-containing peptides as described above. Peptide mass tolerance was set at 0.1 Da, and MS/MS tolerance was set at 0.05 Da. The significance threshold was set to 0.01 resulting in a threshold score of 34. 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 (4,506 proteins, 1,426,768 residues, release 14.4, November 4, 2008, UniProt consortium). False positive rates were estimated as described above and were found to be less than 3%. TCEP-induced reaction products were selected manually, and selected queries were used to recalculate protein coverage and protein score based on azhal-labeled peptides only. The resulting MASCOT data file of this search was imported into Quant (36) for quantitation using the iTRAQ reporter ions using only labeled peptides unique to each protein. For identification purposes both searches were also exported as comma-separated value files. Quant settings were as follows: all four iTRAQ reporters on, report peak areas on, reporter tolerance set at 0.1 Da, intensity range turned off, peak dimensions at 0.025 Da, absolute intensity error set at 0, experimental error set at 0%, use of unique peptides on, *p* value cutoff set at 0.01, and macro language set at English with the macro parameter separator set to comma. The correction factors were put in for iTRAQ kit number 080591. Quant output is a tab-delimited text file containing both reporter ion ratios per peptide as well as mean protein ratios derived from these. To assess the combined effect of technical and biological variance the average and S.D. of all protein ratios of 116/114 and 117/115 for the data set were calculated. These ratios represent replicate B/replicate A at 37 °C and replicate B/replicate A at 44 °C and should theoretically be 1. The

116/114 ratio yielded an average of 1.01 with S.D. of 0.19, and the 117/115 ratio yielded an average of 1.03 with S.D. of 0.20, which shows that no large systemic error was made during mixing of labeled samples. Subsequently tandem MS spectra were inspected manually. Peptides that did not have signals for all four reporter ions or peptides that showed inconsistent 115/114 and 117/116 ratios (biological plus technical replicates) were discarded. Box plots per protein were checked to identify outliers in the peptide ratios per protein as described previously (36). Only nine of 1234 peptides were discarded as outliers. Because of the incorporation of replicates into one quantitation experiment, each peptide can yield four relevant reporter ratios: first of all 115/114 and 117/116 for each prelabeling culture split into two different growth temperatures during labeling and furthermore 117/114 and 115/116 for one growth temperature of one prelabeling culture compared with the other growth temperature of the other prelabeling culture during labeling. For each protein the mean expression ratio was determined by calculating the mean of the peptide ratios 115/114, 117/114, 115/116, and 117/116 per protein (mean ratio of expression between the biological replicates). The accuracy of the ratio per protein is expressed by calculating the S.D. of the peptide ratios for all the peptides measured per protein (maximum S.D. between the biological replicates). To ascertain whether up- or down-regulation was significant, a double sided Student's *t* test for each protein was performed to see whether the mean protein ratio differed significantly from the mean ratio obtained for unregulated proteins ( $\mu = 1.0$  and  $\sigma = 0.2$  from ratio of 116/114 and 117/115). Proteins with a *p* value  $< 0.001$  were considered to have a significantly altered expression level.

**Annotation of Proteins to GO Terms**—To annotate the identified (newly synthesized) proteins with Gene Ontology terms, the list of proteins was assigned using GoMiner (37) run locally with a Derby database engine using the UniProt database and the organism set at 562 (*E. coli*) and evidence codes set at “all.” Resulting GO annotation categories and the corresponding proteins were exported to Excel. With the use of the generic GO-SLIM set (Gene Ontology Consortium) from which strictly eukaryotic terms were removed, the proteins detected were assigned to parent GO categories to analyze the distribution of the newly synthesized proteins over specific biological processes and cellular localizations. To assess the relative over- or under-representation of mapped proteins per category, the number of mapped proteins per category was divided by the sum of the mapped proteins of all the categories in the table. This was done both for categories representing biological processes and cellular localizations as well as for both the entire proteome and the newly synthesized protein data set. Next the relative ratio of representation was calculated for all these categories by dividing the percentage per category for the data set by the percentage per category of the proteome. Thus, ratios greater than 1 indicate relative over-representation of mapped proteins in the category of the newly synthesized proteins compared with the proteome, and ratios smaller than 1 represent relative under-representation.

## RESULTS

**Growth Rate and Viability of *E. coli* Cultured on Azhal**—*E. coli* has previously been shown to efficiently incorporate azhal into recombinant proteins (8–16). However, for pulse labeling applications under relevant physiological conditions it is important to know how *E. coli* grows on azhal and incorporates it into cellular proteins. The  $k_{cat}/K_m$  of methionyl-tRNA synthetase for azhal is 390 times lower than for methionine (11). So no residual methionine should be present during labeling to ensure maximum efficiency of label incorporation.

Therefore the methionine auxotroph MTD123 (31) was used. A range of concentrations of azhal from 10 to 1000 mg/liter was tested (supplemental Fig. 1). Both the growth rate and the growth yield (*i.e.* final  $A_{600}$ ) were maximal at azhal concentrations of 250 mg/liter and higher. For further experiments a concentration of 400 mg/liter of azhal was chosen.

When *E. coli* cells are growing rapidly, incorporation of radiolabeled compounds into proteins is closely related to growth rate (38). Thus, the degree of azhal labeling can be estimated by the increase in cell number as measured by optical density. For the first 30 min after inoculation cells grew with a doubling time on azhal similar to that on methionine (Fig. 2a). In addition, the amount of cellular protein per  $A_{600}$  was also similar for cells grown on azhal (Fig. 2b). After 30 min there is a marked decrease in growth rate of the cells growing on azhal. These cells, after having completed more than one doubling at the reduced growth rate, gradually entered stationary phase after more than 5 h (data not shown). This demonstrates that the growth rate and increase in protein content in the presence of azhal are similar to those of cells grown on methionine but only for the first 30 min upon incubation of cells with the methionine analogue.

To investigate whether cells are still viable after labeling with azhal, samples were taken during growth. The number of viable cells per unit of  $A_{600}$  remained constant between cells grown on azhal and methionine up to 30 min (Fig. 2c). The number of viable cells grown on azhal decreased after 1 h of labeling (supplemental Fig. 2). Toxic side effects not related to azhal incorporation into proteins was tested by growth of cells on a mixture of azhal and methionine. The growth rate was compared with cells grown on methionine only (Fig. 2a). Because of the lower efficiency of the charging of azhal to tRNA by the methionyl-tRNA synthetase, incorporation into proteins is unlikely. Growth rate on the mixture was comparable to that on methionine alone, so no toxic side effects of azhal are apparent from these experiments.

The work described thus far and below was carried out with the MTD123 strain, but growth characteristics of the wild-type *E. coli* K12 strain suggest that application of azhal pulse labeling is not necessarily confined to auxotrophs. When grown in minimal medium containing methionine and shifted to minimal medium without methionine, *E. coli* K12 cells show a lag phase of several minutes before resuming growth. This is due to the time needed to turn on the methionine biosynthesis pathway and generate enough endogenous methionine for growth. On the contrary, wild-type cells shifted to minimal medium containing azhal resume growth immediately at the same rate for the first 30 min as the auxotrophic strain (results not shown). This suggests that a wild-type *E. coli* strain can be used as well provided that labeling times with azhal are short enough to prevent incorporation of methionine in newly synthesized proteins in the course of the pulse.

**Isolation of Azhal-containing Peptides by COFRADIC**—Although *E. coli* does not sustain long term growth on azhal-

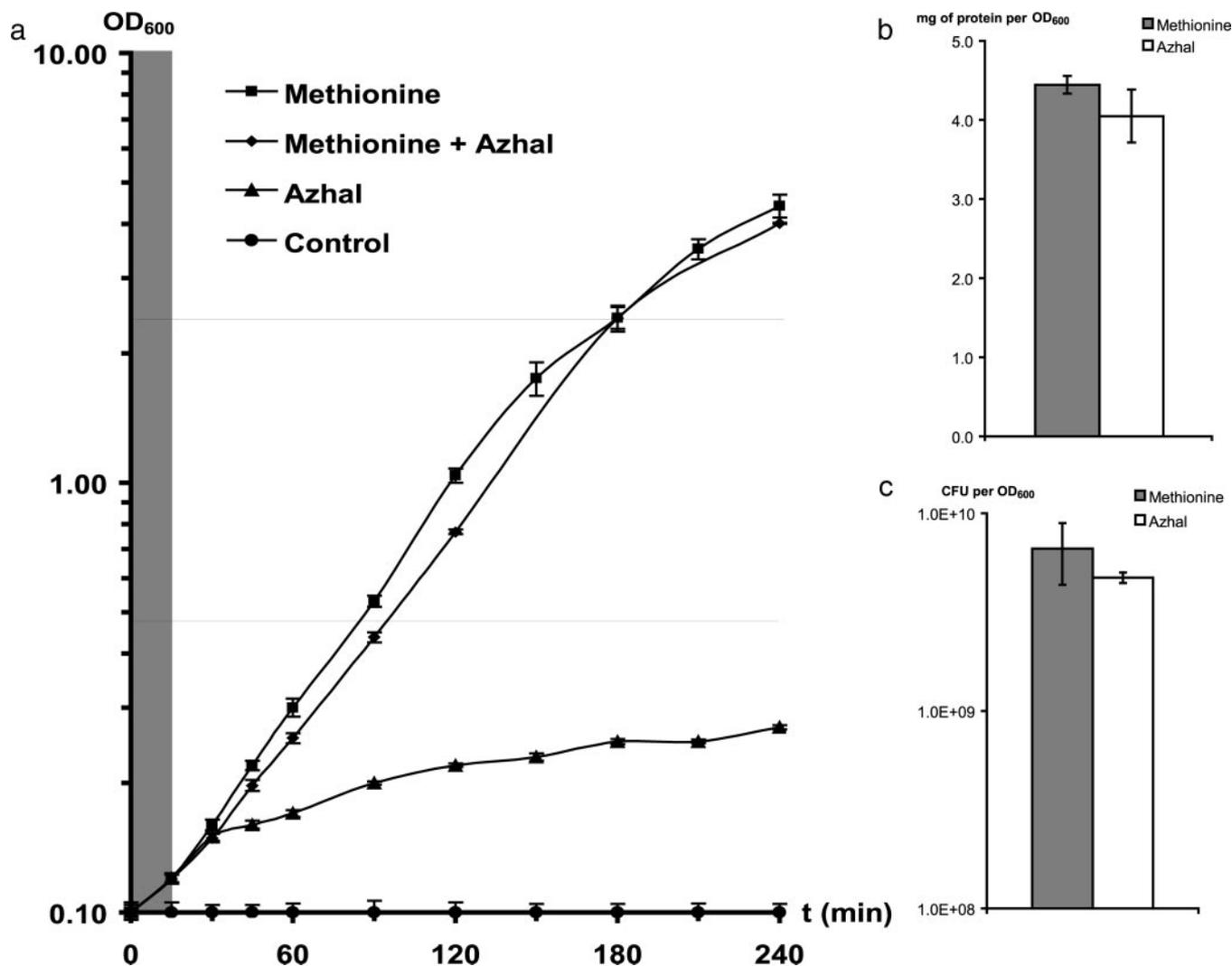


FIG. 2. Growth, viability, and incorporation of azhal in *E. coli*. a, growth curve of *E. coli* strain MTD123 on minimal medium containing either 60 mg/liter methionine, 400 mg/liter azhal, a mixture of 60 mg/liter methionine and 400 mg/liter azhal, or neither as a negative control. Growth rate on methionine alone was  $1.15 \pm 0.04 \text{ h}^{-1}$ . Growth rate on azhal plus methionine was  $1.11 \pm 0.03 \text{ h}^{-1}$ . The gray box shows the time frame used for pulse labeling the cells, error bars indicate S.D. of biological triplicate. The amount of total cellular protein per  $A_{600}$  is shown of cells grown on azhal or methionine (b) as well as the number of colony-forming units per  $A_{600}$  unit for the first 30 min of growth in minimal medium containing methionine or azhal (c). Error bars indicate S.D. of four measurements (biological and technical duplo).

containing media, growth is unperturbed for the first 30 min. Furthermore with no obvious toxic side effects, this amino acid analogue seems well suited to label and identify newly synthesized proteins over short periods of time. To test this, azhal was used to identify proteins synthesized by *E. coli* during a 15-min pulse period in two separate cultures to take biological variation into account. The percentage of labeled protein was estimated to be ~8–9% of total protein after 15 min of growth based on an increase of  $A_{600}$ . After digestion, labeled peptides were isolated using the COFRADIC approach as described under “Experimental Procedures” and depicted in Fig. 1.

The experimental setup is optimized to sequester diaminobutyrate-containing peptides. Peptides in which the azido group has been converted by TCEP into an amine group

generally elute 3–7 min earlier than their parent compound (29). Therefore, they are expected to be collected in the off-diagonal fractions. On the contrary, TCEP-induced cleavage products have a much broader range of retention time differences as compared with their parent compounds and will elute both in on- and off-diagonal fractions. Because of their relatively small size, cleavage products are considered less useful for protein identification purposes than diaminobutyrate-containing peptides. Correct assignment of small peptides by MASCOT is difficult. Moreover small peptides are often singly charged, thereby escaping selection for tandem MS. From the LC-tandem MS data (supplemental Fig. 4) it appeared that in competition with the TCEP-induced reduction and cleavage the azhal residue can also be modified to a homoserine residue where the azide function is substituted by

TABLE I

Number of identified TCEP-induced reaction products

TCEP-induced reaction products in a tryptic digest of an *E. coli* proteome pulse-labeled with azhal were isolated by COFRADIC and identified by LC-tandem MS as described under “Experimental Procedures.” HS, homoserine.

TCEP-induced reaction product	Number of peptides
DAB-containing peptides	641
HS-containing peptides	644
HS- and DAB-containing peptides	73
N-terminal fragment of cleavage at azhal	22
C-terminal fragment of cleavage at azhal	198
DAB- or HS-containing peptides combined with cleavage at azhal <sup>a</sup>	85

<sup>a</sup> DAB- or HS-containing peptides and N- or C-terminal cleavage products derived from the same multiple azhal residue-containing precursor peptide.

a hydroxyl group as indicated by a nominal mass decrease of 25 Da. The proposed reaction scheme of this conversion is presented in supplemental Fig. 5. The relative amounts of each of the TCEP-induced reaction products from a model peptide is depicted in supplemental Table 1.

A total of 2709 peptides were identified in the off-diagonal fractions from the replicate cultures. Of these peptides, 1663 were identified as reaction products derived from azhal-containing peptides, *i.e.* peptides from newly synthesized proteins. Because of the under-representation of cleaved peptides among the TCEP-induced reaction products (supplemental Table 1) and setup of the COFRADIC approach, the number of identified cleavage products is less than the number of identified peptides in which the azhal residue has been converted to a diaminobutyrate or homoserine residue (Table I). The remaining 1046 peptides identified were unlabeled; the presence of unlabeled species in the off-diagonal fractions is probably due to peak tailing of on-diagonal material (Fig. 1c). The contribution of non azhal-containing peptides in the off-diagonal pools is minimized by discarding both the on-diagonal fraction and three adjacent fractions at the front and back. Discarding even more fractions would lead to loss of shifted reaction products. Apart from enrichment of azhal-labeled peptides, COFRADIC has the additional benefit that it provides chromatographic separation during the primary run. Hereby COFRADIC facilitates mass spectrometric identification. Most peptides are only identified in one or two of the 16 LC-MS runs (supplemental Fig. 7).

*Newly Synthesized Proteins Identified by Azhal Labeling—*

From the two biological replicates 527 proteins were identified in single tandem MS runs of the COFRADIC fractions using exclusively reaction products from azhal-containing peptides as described under “Experimental Procedures.” Of these, 294 were found in both replicate cultures. The false positive rate for all proteins identified in the two replicates is under 2%. For

TABLE II

Newly synthesized proteins assigned to Gene Ontology

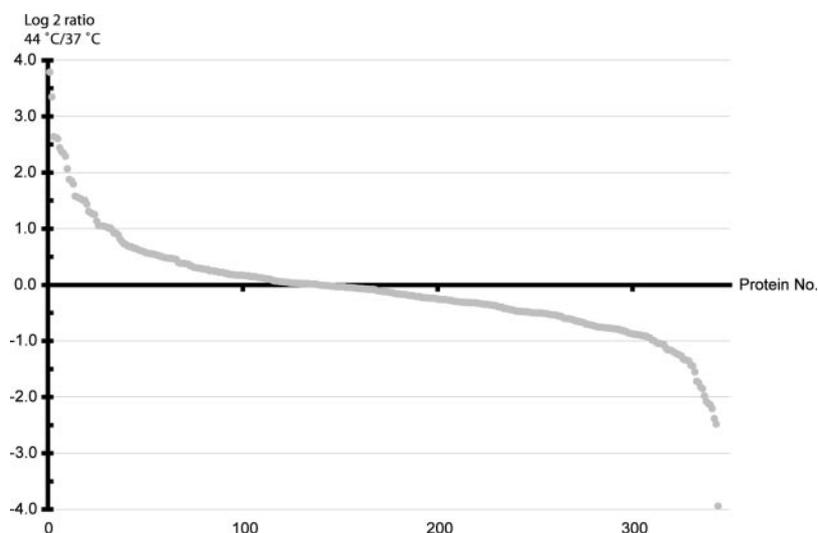
Identified proteins were assigned to Gene Ontology categories of biological processes and cellular localization by GoMiner as described under “Experimental Procedures.”

Description	Number of newly synthesized proteins	Relative ratio <sup>a</sup>
Cell cycle	11	1.2
Signal transduction	16	0.7
Response to stress	12	0.8
Taxis	4	1.1
Transport	66	0.5
Electron transport	27	0.7
Transcription	39	0.7
Translation	74	4.1
DNA metabolic process	17	0.6
RNA metabolic process	72	1.0
Protein metabolic process	115	2.3
Protein catabolic process	1	2.0
Protein modification process	2	0.2
Lipid metabolic process	26	1.0
Secondary metabolic process	10	2.0
Generation of precursor metabolites and energy	44	0.9
Ribosome biogenesis and assembly	5	1.4
Membrane organization and biogenesis	2	2.5
Cell wall organization and biogenesis	5	0.7
Intracellular	162	1.7
Cytoplasm cytosol	138	2.5
Membrane	80	0.4
Periplasmic space	27	0.9
Cell wall	2	0.1
Outer membrane	19	1.1

<sup>a</sup> Relative ratio of the GO annotations per category in the protein data set over those in the proteome. A ratio > 1 indicates a relative over-representation of the category in the data set compared with the proteome; a ratio < 1 indicates a relative under-representation.

proteins identified in both replicates this rate drops to less than 0.5%. To investigate how the proteins synthesized during the 15-min pulse period are distributed according to function and location, they were mapped to their Gene Ontology terms using GoMiner. Of all 527 newly synthesized proteins identified in the two samples, 497 could be mapped to GO terms. The proteins newly synthesized during the pulse period are distributed over all the major categories of Gene Ontology terms present in the *E. coli* proteome (Table II). All major pathways are represented, including energy metabolism, transcription, translation, cell cycle, signal transduction, stress response, and taxis. Furthermore comparison of proteins that are synthesized during the short labeling period to other proteomics studies of *E. coli* (22, 39) grown under comparable conditions in the absence of azhal showed a high overlap in proteins identified (supplemental Fig. 8). This demonstrates again that normal cellular translation of proteins involved in the major cellular pathways continues in the pres-

**FIG. 3. Relative abundance of newly synthesized proteins.** A plot of relative abundance of newly synthesized proteins of cells grown at 44 °C compared with those grown at 37 °C during a 15-min pulse labeling period with azhal is shown.



ence of azhal and that azhal is incorporated into these proteins during pulse labeling.

To isolate membrane proteins additional extraction methods besides urea extraction are needed. In our approach, no such additional extraction steps were used. Thus an underrepresentation of membrane proteins is expected in our data set. Table II shows that indeed this is the case. The relative number of membrane proteins identified to the total number of identified proteins is comparable to the number found by Gevaert *et al.* (22) with a protein extraction protocol similar to that used here (supplemental Fig. 8 and supplemental Table 2). In another study more membrane proteins were identified because of a separate extraction of membrane proteins from cellular debris obtained after extracting soluble protein (39).

**Quantitation of Newly Synthesized Proteins Induced by Heat Shock**—We next studied whether pulse labeling with azhal can be applied on a proteomic scale to determine differences in the amounts of proteins synthesized during a brief time frame upon a change in growth conditions. As a model system for these studies we chose the heat shock response in *E. coli* that has been studied in detail and therefore seemed to be a good system to validate our approach (40–43). Heat shock in *E. coli* is defined as the cellular response to an increase in growth temperature and is accompanied by the up-regulation of a defined set of “heat shock” proteins. The increase of heat shock proteins is controlled by *rpoH*, which encodes the heat shock transcription sigma factor  $\sigma^{32}$  (44–47). Nearly 100 genes have been identified to be part of the  $\sigma^{32}$  regulon (48, 49) of which some 18 genes encode chaperones and proteases. The up-regulation of both chaperones and proteases seems to be aimed at restoring impaired protein folding at higher temperature and to degrade misfolded proteins. The intracellular concentration of  $\sigma^{32}$  shows a rapid transient increase upon heat shock followed by a decrease to reach a new steady state within 10–15 min (50–52). This affects transcription of  $\sigma^{32}$ -regulated genes con-

comitantly and also induces expression of heat shock proteins within this time frame (50, 51). In addition the transcription of a large number of other genes is rapidly up- or down-regulated upon heat shock (53, 54).

To detect candidate proteins whose synthesis rate may change during the transition period we chose a labeling time of 15 min and labeled cells grown at different temperatures with azhal. We used iTRAQ (33) to detect changes in the amounts of proteins synthesized during this brief period by quantitative mass spectrometry. After the 15-min pulse with azhal at either 37 or 44 °C, cells were harvested, and proteins were extracted and digested as described above. The different digests were treated with different iTRAQ labels and then mixed in equivalent amounts. Newly synthesized proteins were identified by azhal-labeled peptides only, enriched by COFRADIC. A total of 394 newly synthesized proteins were identified after the 15-min pulse. Of the identified newly synthesized proteins, 344 could be quantified using the iTRAQ reporter ions according to the criteria formulated under “Experimental Procedures.” Upon an elevation in temperature the relative abundance of 66 newly synthesized proteins significantly ( $p < 0.001$ ) increased or decreased more than a factor of 2 (1 on  $\log_2$  scale), whereas 92 changed significantly ( $p < 0.001$ ) by only a factor of 1.5–2 (0.58–1 on  $\log_2$  scale). The relative abundance of the remaining 186 newly synthesized proteins changed by less than a factor of 1.5 or did not change significantly at all during the 15-min period after temperature switch (Fig. 3).

Among our data set of 344 proteins, we could identify 15 species for which relative synthesis rates under heat shock conditions have been measured previously (50–52, 55, 56). In all cases our data are remarkably similar with these previous studies. The relative amounts of three proteins of which the relative synthesis rates increased dramatically during heat shock, namely GroEL (CH60) (55, 56), chaperone protein ClpB (CLPB) (50, 55), and  $\sigma^{32}$  (RP32) (50–52), were also highly

TABLE III  
 Highly up-regulated proteins upon heat shock

Gene name	Protein	Relative protein ratio <sup>a</sup>	Transcript ratio <sup>b</sup>
<i>uxuA</i>	Mannonate dehydratase	3.8	7.6
<i>pspA</i>	Phage shock protein A	3.3	28.2
<i>htpG</i>	Chaperone protein HtpG	2.6	33.8
<i>uxaC</i>	Uronate isomerase	2.6	1.8
<i>clpB</i>	Chaperone protein ClpB	2.6	36.5
<i>uxuB</i>	D-Mannonate oxidoreductase	2.4	3.2
<i>dnaK</i>	Chaperone protein DnaK	2.4	58.5
<i>ibpB</i>	Small heat shock protein IbpB	2.3	327.5
<i>mopA</i>	60-kDa chaperonin GroEL	2.3	37.9
<i>ybeD</i>	UPF0250 protein YbeD	2.1	1.6
<i>relB</i>	Antitoxin RelB	1.9	3.7
<i>hdhA</i>	7- $\alpha$ -Hydroxysteroid dehydrogenase	1.9	2.5
<i>ybdQ</i>	Universal stress protein G	1.8	1.6
<i>htrA</i>	Protease Do	1.6	9.6
<i>hslU</i>	ATP-dependent hsl protease ATP-binding subunit HslU	1.6	10.3
<i>yfiA</i>	Ribosome-associated inhibitor A	1.5	2.3
<i>sdaA</i>	L-Serine dehydratase 1	1.5	23.6
<i>grpE</i>	Protein GrpE	1.5	24.1
<i>yibT</i>	Uncharacterized protein YibT	1.5	ND
<i>htpX</i>	Probable protease HtpX	1.4	36.1
<i>recN</i>	DNA repair protein RecN	1.3	1.7
<i>rpoH</i>	RNA polymerase $\sigma^{32}$ factor	1.3	4
<i>trxC</i>	Thioredoxin-2	1.3	2.4
<i>lon</i>	ATP-dependent protease La	1.3	20.3
<i>rplD</i>	50 S ribosomal protein L4	1.0	-4.1
<i>yfgB</i>	Ribosomal RNA large subunit methyltransferase N	1.0	-1.2
<i>gltA</i>	Citrate synthase	1.0	-3
<i>rpsH</i>	30 S ribosomal protein S8	1.0	-1.4

<sup>a</sup> Relative protein ratio 44/37 °C of proteins synthesized during a 15-min labeling period upon a change in growth temperature.

<sup>b</sup> Relative transcript ratio 50/37 °C as reported by Richmond *et al.* (53). ND, transcript ratio not determined.

increased under our assay conditions. In general the relative amounts of newly synthesized species of the remaining 12 proteins, all involved in protein biosynthesis, were slightly decreased or not changed at all in agreement with the slightly decreased synthesis rates measured previously under heat shock conditions (55). These results strongly indicate that pulse labeling with azhal is a reliable method to detect changes in the amounts of protein synthesized in a brief time frame upon changes in growth conditions.

Among the 24 proteins of which the relative amounts synthesized during the pulse were increased by at least a factor 2.4 (1.3 on log<sub>2</sub> scale), 14 belonged to the group of  $\sigma^{32}$ -regulated heat shock proteins of which the corresponding transcripts increased considerably during heat shock as measured by Richmond *et al.* (53) (Table III). Remarkable is the presence of three proteins (UxuA, UxaC, and UxuB) involved in hexuronide and hexuronate catabolism in the top six proteins of which the amounts synthesized during the pulse of azhal is increased along with a corresponding increase in transcript level. Of the remaining seven proteins in the top 24, the corresponding mRNA levels are likewise increased in six cases and not measured in one case. The strong correlation between our proteomics data and transcriptomics data available in the literature (53, 54), although measured at different

temperature shifts, underscores the importance of regulation of gene expression at the level of transcription of the most elevated proteins under heat shock conditions.

Interestingly the strong correlation between transcript levels and levels of newly synthesized proteins in the group of most up-regulated proteins did not exist in the group of proteins of which the amounts synthesized during the azhal pulse was decreased by a factor of 2.4 or more (Table IV). Of the 19 proteins in this group, seven are reported to have increased transcript levels upon an elevation in growth temperature (53). The large discrepancy between the levels of newly synthesized proteins and corresponding mRNA levels could be explained simply by assuming a large decrease in the stability of these proteins at elevated temperature. However, even more curious is that among these seven proteins no less than five are enzymes of sulfur metabolism. This suggests that coordinate regulation at a post-transcriptional level rather than mere instability of proteins causes the discrepancy between transcript levels and levels of newly synthesized proteins.

In addition four proteins were identified of which the synthesis increases ~2-fold (1-fold on log<sub>2</sub> scale), whereas transcript levels change in the opposite direction. This suggests regulation at a post-transcriptional level as well (Table III).

TABLE IV  
Highly down-regulated proteins upon heat shock

Gene name	Protein	Relative transcript ratio <sup>a</sup>	Protein ratio <sup>b</sup>
<i>cysM</i>	Cysteine synthase	-1.3	-1.6
<i>purA</i>	Adenylosuccinate synthetase	-1.3	-4.4
<i>b1680</i>	Cysteine desulfurase	-1.3	2.5
<i>rho</i>	Transcription termination factor Rho	-1.3	-7.3
<i>serA</i>	D-3-Phosphoglycerate dehydrogenase	-1.4	ND
<i>tyrB</i>	Aromatic-amino-acid aminotransferase	-1.4	-2.4
<i>cysK</i>	Cysteine synthase A	-1.4	-3.8
<i>yihK</i>	GTP-binding protein TypA/BipA	-1.6	-4.6
<i>rplN</i>	50 S ribosomal protein L14	-1.7	-1.7
<i>cysN</i>	Sulfate adenylyltransferase subunit 1	-1.7	2.3
<i>gcd</i>	Quinoprotein glucose dehydrogenase	-1.8	2.2
<i>cysJ</i>	Sulfite reductase flavoprotein $\alpha$ -component	-1.9	1.3
<i>nlpA</i>	Lipoprotein 28	-2.0	0
<i>rplY</i>	50 S ribosomal protein L25	-2.1	0.1
<i>cysD</i>	Sulfate adenylyltransferase subunit 2	-2.1	2.9
<i>ydfY</i>	Protein GnsB	-2.1	-1.2
<i>hlpA</i>	Chaperone protein Skp	-2.2	-4.8
<i>b2379</i>	Uncharacterized aminotransferase YfdZ	-2.4	1.3
<i>oppA</i>	Periplasmic oligopeptide-binding protein	-2.5	-4.6
<i>cysP</i>	Thiosulfate-binding protein	-3.9	1.9

<sup>a</sup> Relative protein ratio 44/37 °C of proteins synthesized during a 15-min labeling period upon a change in growth temperature.

<sup>b</sup> Relative transcript ratio 50/37 °C as reported by Richmond *et al.* (53). ND, transcript ratio not determined.

#### DISCUSSION

A new method to identify and quantify newly synthesized proteins by the use of azhal as a pulse label is presented. The suitability of azhal as a pulse label was tested by measuring growth and viability of *E. coli* grown on a medium containing azhal as a substitute for methionine. Growth rate and viability in the presence of azhal and methionine was the same during the first 30 min. However, after prolonged labeling growth arrest did occur. This can be explained by assuming dysfunctionality of essential proteins that have one or more crucial methionine residues. No evidence was obtained for toxicity of free azhal that is not incorporated into proteins. A direct toxic effect of azhal on the cells might cause an immediate stress response; however, such a response seems to be absent. An important prerequisite for a pulse label is that it does not cause major changes in protein expression during the labeling period. The labeled proteins should be representative of the translational activity of the cell at the start of the pulse. No major differences were found between our data set of newly synthesized proteins and the lists of proteins identified by others (22, 39) in *E. coli* grown under comparable conditions in the absence of azhal. Moreover there is evidence for normal protein processing of the N-terminal azhal residue in *E. coli* (57). In addition, evidence for normal localization and folding of azhal-labeled proteins comes from the observation that the membrane protein OmpC is displayed at the cell surface of *E. coli* (12, 13, 15), and recombinantly produced virus-like particles assemble normally (16) when methionine is replaced by azhal. These findings in combination with the fact that over 500 proteins are found to be labeled, representing

all major pathways in the cell, lead us to believe that azhal is a suitable label for pulse-chase experiments in *E. coli*.

We used pulse labeling with azhal to determine the relative abundance of proteins synthesized during the transition period after a change in growth temperature from 37 to 44 °C. By the use of iTRAQ for relative quantitation of newly synthesized proteins, the relative abundance of 344 proteins was determined upon a change in growth temperature. Among proteins highly up-regulated after the temperature switch there were many heat shock-inducible chaperones and proteases, all part of the  $\sigma^{32}$  regulon. Levels of proteins for which radiolabeling data were available showed high similarity to levels of newly synthesized proteins determined by azhal labeling. This further validated that the protein expression measured here is due to temperature-induced changes in gene expression and not the result of labeling cells with azhal.

Comparison of the results with transcript data from the literature revealed that many proteins that are highly up-regulated upon a temperature switch seem to be regulated at the transcriptional level as increased transcript levels corresponded with an increase of protein synthesis as determined by azhal incorporation. Examples to illustrate this are the genes *uxuA*, *uxuB*, and *uxaC* that encode enzymes that catalyze different steps in the catabolism of hexuronides and hexuronates to 2-keto-3-deoxygluconate. 2-Keto-3-deoxygluconate is metabolized by the Entner-Doudoroff pathway and enters the lower part of glycolysis (58). There are no previous reports about temperature induction at the protein level of members of this pathway. However, UxuA, UxuB, and UxaC were found to be highly up-regulated upon heat shock

in this study in good correlation with altered transcript levels reported before (53). A possible explanation for the measured increase in transcription could be instability of the transcriptional repressors of these genes (ExuR and UxuR) at higher temperatures. In contrast to the highly up-regulated heat shock proteins, there was poor correlation between transcript data and relative levels of newly synthesized proteins in a group of highly down-regulated proteins. Increased turnover of these proteins at higher temperature offers one explanation. However, several proteins in this group are involved in the same metabolic niche of sulfur metabolism, raising the interesting question whether coordinate regulation at the level of translation may take place. Further studies aimed at determining actual relative translation and degradation rates are needed to enable formulation of hypotheses concerning the molecular events underlying the discrepancy of transcript levels and levels of newly synthesized proteins for this group of genes. Also for a group of four proteins found to be up-regulated with transcript levels going down significantly upon elevation of the growth temperature, post-transcriptional regulation is a possible explanation. This shows that azhal labeling is very suitable to identify candidates that may be subject to post-transcriptional regulation.

Up to now pulse labeling has been shown for organisms that are either naturally auxotrophic for methionine, *i.e.* mammalian cells (20), or in this study with a genetically engineered methionine-auxotrophic *E. coli* strain. The requirement to use an auxotrophic organism could limit the application of pulse labeling with azhal to natural auxotrophs and organisms for which an auxotroph can be obtained through genetic manipulation. However, growth of the wild-type *E. coli* K12 strain suggests that bioorthogonal pulse labeling of proteins using azhal is not necessarily confined to auxotrophs. In general, the use of azhal as a pulse label in any prospective organism first and foremost depends upon favorable growth and incorporation characteristics of azhal and should therefore always be tested for each organism individually.

The COFRADIC-based method to isolate labeled peptides is an alternative to using affinity purification as described by Dieterich *et al.* (20). Our approach, however, has several advantages. First of all, it utilizes standard chromatographic techniques and chemicals to achieve separation of labeled from unlabeled peptides. Secondly it provides chromatographic fractionation as well as enrichment of labeled peptides, thereby facilitating mass spectrometric identification. Furthermore it provides a simple and robust approach to enrichment of labeled peptides. The TCEP-based chemistry is compatible with a range of pH values, temperatures, and buffer compositions and has been proven to be compatible with iTRAQ labeling. The application of extraction protocols geared toward membrane proteins should give this approach the ability to measure the changes in synthesis of this class of proteins as well. We show that only a short labeling time is required for relative quantitation of hundreds of newly synthe-

sized proteins. It should be noted, however, that there may already be a significant contribution of degradation to the amount of each protein formed in 15 min. This is dependent on the half-life of the particular protein. Shorter labeling times may give more accurate translation rates. This, however, could be at the expense of the number of quantifiable proteins.

Here we clearly demonstrate the power of azido peptide isolation by COFRADIC to sequester labeled peptides by the large number of newly synthesized proteins identified. Also the first proteome-wide approach to quantitation of newly synthesized proteins by azhal labeling is demonstrated here using COFRADIC. Altogether the fact that no severe azhal-related disturbances were obvious combined with the large number of newly synthesized proteins identified and quantified makes azido peptide isolation by COFRADIC in combination with iTRAQ an excellent tool for both identification and quantitation of transient changes in protein expression. The pulse labeling technique described is uniquely suited to follow an adaptation to changes in the environment of *E. coli*. Furthermore comparison with transcript data allows for screening for different types of regulation in response to a change in environment. The presented method can open up new avenues in systems biology research by filling the gap of information between transcriptomics and proteomics and allow for new input into advanced modeling of cellular networks.

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¶ Present address: Pepsican Therapeutics BV, Zuidersluisweg 2, 8243 RC Lelystad, The Netherlands.

‡‡ To whom correspondence should be addressed. Tel.: 31-20-525-5457; Fax: 31-20-525-6971; E-mail: C.G.DeKoster@uva.nl.

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