Deuterium induces a distinctive *Escherichia coli* proteome that correlates with the reduction in growth rate

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Substitution of protium (H) for deuterium (D) strongly affects biological systems. Whereas higher eukaryotes such as plants and mammals hardly survive a deuterium content of >30%, many microorganisms can grow on fully deuterated media, albeit at reduced rates. Very little is known about how the H/D replacement influences life at the systems level. Here, we used MS-based analysis to follow the adaptation of a large part of the *Escherichia coli* proteome from growth on a protonated full medium, over a protonated minimal medium, to a completely deuterated minimal medium. We could quantify >1800 proteins under all conditions, several 100 of which exhibited strong deuterated minimal medium. We could quantify >1800 proteins under all conditions, several 100 of which exhibited strong deuterated minimal medium. We could quantify >1800 proteins under all conditions, several 100 of which exhibited strong deuterated minimal medium. We could quantify >1800 proteins under all conditions, several 100 of which exhibited strong deuterated minimal medium. We could quantify >1800 proteins under all conditions, several 100 of which exhibited strong deuterated minimal medium. We could quantify >1800 proteins under all conditions, several 100 of which exhibited strong...
lipids by about 1 K (20) and usually, but not always, increases the thermal stability of proteins (21–28). In contrast, covalent deuteration reduces the thermal stability of proteins (21, 29). Interestingly, deuteration of phospholipids also decreases the temperature of the gel to liquid crystalline phase transition by 4–5 K and lipid structural order (30, 31). These observations for both proteins and lipids hint at a reduction of attractive van der Waals interactions under deuteration. Compared with these moderate changes in stability, the stronger kinetic isotope effects, i.e. the reduction of hydrogen transfer rates (15, 17), are likely to be more critical for cellular function, but comprehensive studies are lacking and descriptions have remained largely phenomenological.

Early microscopic observations suggested that D2O affects the size and morphology of cells and microorganisms (1, 2, 4) and chloroplast ordering in algae (1) and causes mitotic arrest (4, 32), which in microorganisms may be overcome by adaptation (4). Most of these observations have not been analyzed further by modern high-resolution techniques. Of note, the inhibition of mitosis has been investigated by immunofluorescence microscopy (33) and in vitro assays of isolated microtubules (34). The latter show increased microtubule polymerization and decreased rates of microtubule growth and GTP hydrolysis in D2O (34). On the level of general cellular functions, a reduction of protein translation and an accumulation of translation and decreased rates of microtubule growth and GTP hydrolysis in D2O (34). On the level of general cellular function (33) and inhibition of mitosis has been investigated by immunofluorescence microscopy (33) and in vitro assays of isolated microtubules (34). The latter show increased microtubule polymerization and decreased rates of microtubule growth and GTP hydrolysis in D2O (34). On the level of general cellular functions, a reduction of protein translation and an accumulation of translation and decreased rates of microtubule growth and GTP hydrolysis in D2O (34). On the level of general cellular function (33) and inhibition of mitosis has been investigated by immunofluorescence microscopy (33) and in vitro assays of isolated microtubules (34).

Results
Adaptation of E. coli to growth in a deuterated medium
To study the effect of a deuterated environment on E. coli under well-controlled conditions, we compared growth on protonated and deuterated minimal media, which can be easily generated from D2O and deuterated glucose as the sole hydrogen sources (besides trace amounts of vitamins and EDTA). Very reproducible adaptation of E. coli to such a highly deuterated (≥99%) environment was achieved in a stepwise manner (Fig. 1). Cells were initially seeded in protonated full (LB) medium and then transferred sequentially to protonated M9 minimal medium (M9H1), 70% D2O M9 medium (M9D2), and finally perdeuterated M9 medium (99.8% D2O, 97% deuterated glucose-δ7, M9D3). At each step, cells were grown within the exponential phase to an A600 of about 0.3–0.6 for typically five generations. For a stringent comparison, protonated M9H3 cultures were also generated as two consecutive cultures in protonated M9 medium from the M9H1 cells to match the total number of generations to the M9D3 cultures. To study the influence of the growth phase, samples for analysis were taken both from the exponential phase as well as from an extended cultivation of the cells to the early stationary phase (A600 ≥ 1.2, Table S1). The transfer to the minimal and deuterated medium increased the growth rate ( = ln(2)/generation time) from 1.8 ± 0.2 h−1 (LB), over 0.67 ± 0.02 h−1 (M9H1 and M9H3) to 0.37 ± 0.01 h−1 (M9D3). The nearly 2-fold decrease in growth rate in the deuterated medium provides manifest evidence of the severe deuterium isotope effects on E. coli cellular functions.

Cell shape analysis by light microscopy and cryo-EM
As early reports indicated strong changes in morphology and cell size of microorganisms induced by D2O (1, 4), we sought to investigate this phenomenon by modern microscopic techniques. Cells from exponentially grown M9H3 and M9D3 cultures were indistinguishable with respect to their general appearance by phase-contrast light microscopy (Fig. 2A). A quantitative analysis (Fig. 2B) revealed a slight decrease in mean length (2.60 ± 0.02 μm → 2.41 ± 0.02 μm) and a slight increase...
in mean width (0.764 ± 0.003 μm → 0.823 ± 0.004 μm) because of the change from protonated to deuterated medium. These changes are minor relative to the observed variations of length and width within the cell populations (0.4 μm, 0.1 μm). To detect changes at higher resolution, we also analyzed plunge-frozen samples by cryo-EM. For both cultures, inner membrane, peptidoglycan layer, as well as the outer membrane were distinctly observable in the micrographs (Fig. 2 C), but again indicated a very similar overall organization and size of the cell envelope in M9D3e compared with M9H3e cultures. Also, no particular differences are observable in the cellular interior and its visible ribosomal particles. Thus our results, in contrast to the early literature reports, indicate that growth on the deuterated medium does not induce strong effects on shape and cellular organization of E. coli.

Proteomics data quality and biological variability

To follow the adaptation to D2O at a detailed molecular level, we have analyzed the proteome of E. coli grown on protonated full (LB), protonated minimal (M9H3), and deuterated minimal (M9D3) media using shotgun LC-MS/MS of proteolyzed samples. A high accuracy of the relative protonated and deuterated protein abundances was obtained by combining protonated and deuterated proteolyzed samples as 1:1 mixtures into single samples for combined LC-MS/MS. The acquired LC-MS/MS data were subjected to a combined search and quantification of protonated and deuterated peptides taking into account the reduced retention times of deuterated peptides yielding a high correlation between the detected protonated and deuterated proteins (r² = 0.859, see “Materials and methods” for details). To verify the accuracy of the detected mass intensity changes, we analyzed different ratios of protonated and deuterated peptide mixtures in the range of 0.1 to 10 (Fig. 3A). The ratios of detected deuterated versus protonated mass intensities showed an excellent linear correlation (r² = 0.9999) with the relative input concentrations of deuterated and protonated peptides. Thus our detection procedure accurately determines relative abundances of deuterated and protonated proteins over at least two orders of magnitude.

In total 1849 proteins were detected in common among all media conditions for samples obtained from the exponential- and also stationary-phase cultures (denoted by the appendices “e” and “s”, respectively). Triplicate, independent cell cultures yielded very good reproducibility of the protein abundances with very similar distributions of their coefficients of variation (CV, relative standard deviations) for all conditions and median CV values in the 10% range (Fig. 3B).

A comparison between the different conditions showed highly significant effects. Thus 496 proteins exhibited at least 2-fold abundance changes with a q-value ≤ 0.01 when moving from the LB medium (LBe) to the protonated minimal medium (M9H3e) (Fig. 3C, left panel). The subsequent adaptation to the deuterated (M9D3e) minimal medium caused similarly strong changes for 384 proteins (Fig. 3C, right panel). Within these sets of regulated proteins, 143 are common between the adaptations from LBe to M9H3e and from M9H3e to M9D3e. Very similar abundance changes were observed between stationary-phase
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![Figure 3. Linearity test and statistical quality of proteomics data on the *E. coli* adaptation to minimal and deuterated media. A, linearity test of detected deuterated MS intensities in mixed samples of protonated and deuterated *E. coli* peptide solutions. Ratios of summed deuterated and protonated detected intensities MS(D)/MS(H) are plotted against the ratio [D]/[H] of protonated and deuterated input peptide concentrations. The dashed line corresponds to a linear fit of all points with MS(D)/MS(H) = 0.475 [D]/[H] (r² = 0.9999). B, coefficients of variation (relative standard deviations, CVs) of the protein abundance data. C, log₂ fold changes in protein abundance and their statistical significance (q-value) in the proteomes of *E. coli* cells grown in protonated M9 minimal versus protonated LB medium (M9H3e/LBe, left panel) and deuterated versus protonated minimal medium (M9D3e/M9H3e, right panel), respectively.

LBs and M9H3s as well as M9H3s and M9D3s cultures. The intensities and statistics of all detected proteins are listed in Table S2.

**Principal component analysis reveals unique proteomes in full, minimal, and deuterated media**

A principal component analysis of the total variations of protein abundances reveals that all three conditions of full (LB), protonated minimal (M9H3), and deuterated minimal (M9D3) media are separated into distinct clusters within the first and second principal components (Fig. 4). The high reproducibility within the triplicate cell cultures indicates a uniform and robust adaptation to the different conditions. Variations between exponential- and stationary-phase cultures are also small within both minimal media conditions (M9H3e/s, M9D3e/s), but are larger for the protonated full medium (LBe/s). Interestingly the position of LBs within the first two principal components is located on a line from LBe toward M9H3e/s at about one fifth of their distance. This points at a nutrient limitation under the stationary LB conditions, which is similar to, but not yet as severe as in, M9 medium. In strong contrast, the positions of M9D3e/s are clearly not collinear with the line connecting LBe and M9H3e/s. Thus the proteome changes because of deuteration are highly distinct from those caused by the nutrient limitations of the minimal medium.

![Figure 4. Principal component analysis of normalized protein abundances for different growth media. The proteome abundance vectors of *E. coli* grown in protonated full LBe/s, protonated (M9H3e/s), and deuterated M9 minimal (M9D3e/s) media strongly differ in their first two principal components, but are similar between respective exponential (e) and stationary (s) cultures.

**Overall proteome resource allocation**

Because the total of proteins detected by the present mass spectrometric analysis covers almost the entire cellular expressed protein content (37), the protein MS signal/total MS signal is a very good estimate of the cellular protein mass/total cellular protein mass (38). To obtain an overview of the proteome allocation to different functional categories, we summed the protein signals detected within the 22 categories of the Clusters of Orthologous Groups (COG) database (39) and normalized these values by the total detected MS signal. Across each condition, the largest fraction of the detected proteome belongs to the COG translation category J, which comprises the ribosomal proteins and amounts to 36% of the total protein mass for LBe (30% for LBs) (Fig. 5A). Upon change to protonated minimal medium (M9H3e/s), this fraction is reduced to 27%, and the second largest category E associated to amino acid metabolism is up-regulated to 13% from the 7% in LB. This is consistent with the absence of amino acids in the minimal medium, which need to be synthesized by *E. coli*. However, the reduced supply of amino acids apparently still limits translation and in consequence reduces the amount of proteins in category J. Similar-sized variations of this category in response to nutrient supply have been observed previously (37, 40). For instance, as compared with a glucose minimal medium, 9% of protein mass in *E. coli* was saved on amino acid synthesis in an LB medium and allocated to translation, which made the growth rate jump from 0.65 to 1.6 h⁻¹. In fact, a very good linear relation exists between the protein amount in category J and the growth rate where a 5% increase in J approximately increases the growth rate by 0.5 h⁻¹ (37).

Compared with the protonated minimal medium (M9H3), the deuterated minimal medium (M9D3e/s) causes a further reduction of proteome allocation to the translation category J from 27 to 22%, which is again consistent with the observed further decrease in the cellular growth rate. Indeed a comparison of the proteome allocation to category J to observed growth rates in LBe, M9H3, and M9D3 (Fig. 5B) exactly reproduces the previously obtained linear relation (37). Only very minor variations occur in the other COG categories upon change to the
deuterated medium. In particular, no significant change is observed for the amino acid metabolism (category E) proteins. Thus translation and growth under deuteration are apparently not limited by amino acid synthesis, otherwise one would expect an up-regulation of category E. Rather, translation and growth must be limited by other pathways.

Changes of the proteome specific to minimal medium

To identify specific cellular responses to the growth media, we have selected significantly regulated proteins from the total observed proteome changes in two different ways: (i) we selected the set of 30 most strongly regulated, individual proteins (set S1) and (ii) we clustered all detected proteins into functionally related classes according to the Gene Ontology (GO) database and identified the most strongly regulated classes. For a robust identification of the latter, only GO classes were considered, for which at least 75% of their contained proteins were detected with such low variation that they could be classified as either strongly regulated (at least 2-fold up or down) or as not strongly regulated (less than 2-fold). The entire GO class was then considered as strongly up- or down-regulated when at least 50% of its significantly detected proteins were at least 2-fold up- or down-regulated, respectively. A superset of significantly regulated proteins (set S2) was then created by merging set S1 with the more than 2-fold regulated proteins in the strongly regulated GO classes. Fig. 6 shows the set S1 of the 30 most strongly regulated proteins for the change from the LBe to the M9H3e minimal medium (Fig. 6A), a selection of the significantly regulated GO classes (Fig. 6B), and a histogram of set S2 according to the COG categories (Fig. 6C). All identified significant GO classes and the superset S2 of 154 significantly regulated proteins are given in Fig. S1 and Table S3, respectively. Note that here and in the following, only data from the exponential growth cultures were considered to exclude effects of nutrient and oxygen limitation in the stationary phase.

Consistent with the lack of amino acids, many of the most strongly up-regulated proteins in the minimal medium M9H3e (Fig. 6, A and C) are part of the COG amino acid metabolism category E and comprise proteins involved in biosynthesis of branched amino acids ( livJ, livK, ilvN, leuA, gene names are used for clarity), lysine ( lysC), methionine ( metE, metF), tryptophan ( trpD), binding of glutamate/aspartate ( gltI), as well as peptide chemotaxis ( dppA) (41). Conversely, proteins associated with amino acid degradation and import ( sdaB, sdaC, tdcG) as well as peptide transmembrane ( dtpA) and amine betaine ( prov) transport are strongly (>10-fold) down-regulated.
Figure 6. Functional analysis of proteome changes between \textit{E. coli} cells grown in protonated M9 minimal (M9H3e) and protonated full (LBe) medium. A, log2 fold changes of the 30 most strongly regulated proteins and their assigned COG classes (in parentheses). B, selection of significantly regulated (\textit{red}: up; \textit{blue}: down) or significantly not-regulated (\textit{black}) protein clusters identified by the GO classification (see text). The values in the last line of each GO class indicate from left to right: the number of significantly up-regulated (Nup), significantly not regulated (Nnot regulated), significantly down-regulated (Ndown), not significantly detected (Nnot significant), as well as the total number of proteins in the class (Ntot). The complete set of identified significant GO classes is shown in Fig. S1. C, functional distribution of the superset S2 of strongly regulated proteins (see text) according to their COG classification.
The increase in amino acid synthesis is also very clearly visible in the very strong up-regulation of many GO classes (Fig. 6B) associated with de novo amino acid biosynthesis (cellular amino acid biosynthetic process, GO:0006652; aromatic amino acid family process, GO:0000972; and their subclasses). Conversely, proline transport (GO:0015193 and GO:0015824) is strongly down-regulated. Interestingly, 45 of the 58 proteins of the GO class assigned to the ribonucleoprotein complex (GO: 1990904) could be quantified reliably. All of them are less than 2-fold regulated, which is consistent with the moderate down-regulation of the total COG translation category J by about 25% (Fig. 5A).

Also consistent with the fact that glucose is the sole carbon source of the minimal medium, very strong up-regulation is observed for two proteins central to the citric acid cycle (gltA) and its glyoxylate shunt (aceA) (42, 43) (Fig. 6A). Conversely, the strong down-regulation of the membranous glucose dehydrogenase (gcd) is expected to increase the availability of glucose by the repression of its direct oxidation. Corresponding to this importance of glucose and its import from the medium, multiple GO classes assigned to active glucose import are strongly up-regulated (Fig. 6B). Further, notably regulated proteins comprise two strongly up-regulated proteins of the enterobactin biosynthesis pathway (entA, entB), which suggests an increase of siderophore-based iron uptake, as well as strongly down-regulated proteins associated with biofilm formation (yhcN, bhsA) and the associated biosynthesis of cellulose (bcsB) (44, 45). The reported repression of biofilm formation by glucose (46) corroborates this observation.

The discussed proteins are only a small fraction of all proteins regulated upon change to minimal medium. A comprehensive understanding will require detailed follow-up studies. However, the largest changes of the proteome are clearly directed to utilize glucose as an energy source and synthesize amino acids (Fig. 6C). The shortage of the latter apparently limits translation.

Changes of the proteome specific to deuterated minimal medium

The response to the change from protonated (M9H3e) to deuterated (M9D3e) minimal medium markedly differs from the change from full to minimal medium. The respective set S1 of the 30 most strongly regulated proteins (Fig. 7A) and super-set S2 of 120 significantly regulated proteins (Fig. 7C and Table S4) span a much larger variety of functional COG categories than found for the latter. Correspondingly, the identified strongly regulated GO classes (shown as selection in Fig. 7B and in full in Fig. S2) also are highly diverse, albeit the individual classes are rather small and often only very few proteins are affected. Consistent with the moderate overall down-regulation (~20%) of the COG translation category J in the deuterated medium and similar to the findings for the minimal medium, also the well-detected ribonucleoprotein complex GO class is not strongly regulated (Fig. 7B). Thus neither the COG nor the GO classification reveals any single biological mechanism, which is dominantly affected by the deuterated medium.

The change to D$_2$O and deuteration of cellular compounds is expected to slow hydrogen transfer and to affect chemical equilibria as well as the stability of biomolecules and their assemblies. In search for such a connection, we analyzed the super-set S2 of significantly regulated proteins (Table S4) according to their specific function described in the UniProt database. This analysis reveals that indeed most of the regulated proteins can be linked to these expected physicochemical effects. Many of them are associated with (i) hydrogen transfer reactions, (ii) redox reactions, or (iii) membrane transport. A further significant number of regulated proteins are involved in (iv) nucleic acid metabolism and (v) homeostasis, which is not immediately apparent from the COG and GO classifications. Histograms of the number of up- and down-regulated proteins associated with groups (i–v) are shown in Fig. 7D. The following paragraphs discuss specific findings.

Hydrogen transfer reactions

Hydrogen transfer occurs in all reactions involved in the synthesis or cleavage of polycarbon chains as well as phosphorylation and dephosphorylation. Important examples comprise glycolysis and glycogen synthesis in the carbohydrate metabolism, most intermediate steps of the TCA cycle, amino acid metabolism, nucleic acid metabolism, ATP-driven energy transfer, as well as phosphosignaling. These reactions are expected to be significantly slowed upon change to a deuterated medium. An increase in the respective enzyme concentrations will counteract this slowing of the reaction rates and may lead to a net compensation of effects.

Of the 120 significantly regulated proteins in set S2, 73 are more than 2-fold up-regulated (Table S4), 57 of which can be clearly associated with hydrogen transfer. Hence 78% of the up-regulated proteins are hydrogen transfer enzymes (Fig. 7D). This is significantly higher than their average of 55% (673 proteins) within the total of 1228 significantly regulated or significantly nonregulated proteins (Table S2). In contrast, the 26 down-regulated hydrogen transfer enzymes constitute only 55% of all 47 down-regulated proteins in set S2, agreeing with their average in the total set of significantly detected proteins. Thus the hydrogen transfer enzymes are significantly up-regulated under deuterated conditions in agreement with the hypothesis of a compensation for their reduced reaction rates.

Examples of the most strongly up-regulated proteins (Fig. 7A) are the sulfur transferase tusB (gene name used for clarity), the sucrose phosphorylase ycjM, the guanosine methylase rlmA, the ATPase and GSH importer gsiA, the phosphotransferase chbB, the phosphodiesterase glpQ, and the transaminase gabT. The inspection of most significantly up-regulated GO classes also reveals hydrogen transfer as the basic reaction of their up-regulated enzymes (Fig. S2 and Table S4). In particular, strong up-regulation occurs for glgA, glgB, glgC, glgP of the GO class glycan metabolic process (GO:0005977, Fig. 7B), which synthesize glucan chains using ADP-glucose, catalyze the formation of glycosidic linkages, synthesize ADP-glucose, or have glycogen phosphorylase activity, respectively. Furthermore, the up-regulated gldA (glycerol metabolic process, GO:0006071) catalyzes the NAD-dependent oxidation of glycerol to dihydroxyacetone, maeA (malic enzyme activity, GO:0004470) is a malate dehydrogenase, dldA (d-alanine-d-alanine ligase activity, GO:0008716) ligates d-alanine under ATP hydrolysis, and fabB
Figure 7. Functional analysis of proteome changes between *E. coli* cells grown in deuterated M9 minimal (M9D3e) and protonated M9 minimal (M9H3e) medium. **A**, log2 fold changes of the 30 most strongly regulated proteins and their assigned COG classes (in parentheses). **B**, selection of significantly regulated (red: up; blue: down) or significantly not regulated (black) protein clusters identified by the GO classification (see text). Several clusters (magenta) also showed significant combined up- and down-regulation \((N_{\text{up}} + N_{\text{down}})/N_{\text{not regulated}} + N_{\text{down}}) > 50\%\) of their detected proteins, albeit they were not significantly regulated in one direction (up or down). The complete set of identified significant GO classes is shown in Fig. S2. **C**, functional distribution of the superset S2 of strongly regulated proteins (see text) according to their COG classification. **D**, relative fractions of up- and down-regulated proteins in the set S2 according to their classification for (i) hydrogen transfer reactions, (ii) redox reactions, (iii) membrane transport, (iv) nucleic acid metabolism and (v) homeostasis (Table S4). The total number of proteins is indicated at the top. The dashed line indicates the fraction of hydrogen transfer enzymes identified in the total set of significantly regulated or significantly nonregulated proteins (Table S2).
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(3-oxoacyl-[acyl-carrier-protein] synthase activity, GO:0004315) elongates fatty acids involving hydrogen transfer of histidine and cysteine residues in its active sites.

**Redox reactions**

Deuterium affects enzyme-catalyzed redox reactions by the KIE of proton-coupled electron transfer as well as by a change of redox equilibria (17, 47, 48). Electron transfer rates in D₂O are often slower (48), but can also be faster (47). Changes in the concentrations of the involved enzymes may counteract these variations. Indeed 16 of the 120 significantly regulated proteins in set S2 are involved in redox reactions, 13 of which are up-regulated (Fig. 7D). We observed e.g. strong up-regulation for the oxidoreductase ndrH and the transporter cydD essential for cytochrome biogenesis (cell redox homeostasis, GO:0045454), the sulfurtransferase subunit tusB, the oxidoreductase yhbW, and the poorly characterized sulfur acceptor ygdK. Conversely, the terminal oxidase cyoD associated with the cytochrome bo₃ complex is strongly down-regulated in the deuterated medium.

**Membrane transport**

The lower phase transition temperature and order of deuterated lipids (31) is expected to increase the fluidity of the deuterated cell membrane, thereby affecting stability and function of integral and membrane-associated proteins. The deuteration of the membrane proteins themselves as well as KIEs of reactions may further influence their function. We find that 10 of the 18 membrane transporters in set S2 are strongly down-regulated under deuteration (Fig. 7D). This includes the down-regulated maeE, maeF, malk (maltodextrin transport, GO:0042956) and the periplasmic mals that are involved in maltoligosaccharide transport, whereas alsB is required for binding and import of D-allose. Strong down-regulation also occurs for the outer membrane-bound ycfJ with unknown biological function. Conversely, e.g. glpF and glpT (polyl transport, GO:0015791) and the peptide transporter mppA are strongly up-regulated.

**Nucleic acid metabolism**

A relatively large number of enzymes involved in nucleic acid metabolism (25 in set S2, Fig. 7D) are differentially regulated in the deuterated medium. Because D₂O solvent causes only a minor increase (~2 K) in the melting temperature of DNA double strands (18, 19), again the KIE of hydrogen transfer reactions is a more likely cause for this regulation. Indeed, with the exception of the poorly characterized ybjX, the activity of all these proteins clearly involves hydrogen transfer reactions. Strong up-regulation is observed for 18 proteins, e.g. for the poly(A) polymerase I pcmB (mRNA polyadenylation, GO:0006378), the exoribonuclease rph (tRNA 3’-end processing, GO:0042780), the RNA helicase deaD (cellular response to cold, GO:0070417), the exonuclease uvrA, and the conserved DNA damage response protein ybjX. Conversely, strong down-regulation was observed e.g. for the exonuclease rnt (tRNA 3’-end processing, GO:0042780) and the helicase recG (Holliday junction helicase complex, GO:0009379), the carboxylase speD, and the propylamine transferase speE (spermidine biosynthetic process, GO:0008295). These changes of nucleic acid processing enzymes are expected to have a significant impact on transcription and translation in deuterated medium, which may correlate to the decreased growth rates.

**Homeostasis**

Not surprisingly, a notable number of proteins (26 in set S2, Fig. 7D) regulated in response to the deuterated medium are associated with homeostasis. Examples comprise the up-regulated trehalose-phosphate synthase otsA, the RNA helicase deaD (cellular response to cold, GO:0070417), and the essential cell cycle regulator engB (49). In contrast, down-regulation is observed for hipA and ycfQ, associated with dormancy and biofilm formation, the heat shock response protein rpoH, and the repressor of gluconate utilization gntR.

**Discussion**

We have shown that adaptation of E. coli from a full medium to a minimal medium and subsequently to a perdeuterated minimal medium can be followed by proteomics on more than 1800 proteins, several hundred of which are significantly regulated in both cases. A consistent up-regulation of proteins associated with amino acid synthesis and glucose utilization occurs in response to the minimal medium concomitant with a 9% overall down-regulation of proteins allocated to the translation category J and a slowing of the growth rate. The response to deuteration is highly distinct from the latter: It affects a much larger variety of proteins and a conventional functional classification by COG and GO annotations fails to identify any dominantly affected cellular mechanism. The total protein abundances in individual COG categories show only minor changes, but the further reduction by 5% of proteins in translation category J coincides with a further reduction in growth rate. The correlation between protein abundance in category J and growth rate reveals a clear linear dependence, which is in excellent agreement with earlier results for various nondeuterated growth media (37). Thus the reduced growth rate in perdeuterated medium and the reduced abundance of translational proteins are obviously connected; however, the cause for the down-regulation of the translational machinery is unclear. Because deuteration does not induce significant changes in the amino acid metabolism category E, the latter is apparently not the limiting factor for growth in the deuterated environment.

An in-depth analysis of the individual, regulated proteins reveals that most up-regulated proteins can be associated with hydrogen transfer and redox reactions, which are expected to be considerably slowed in a deuterated medium by the KIE. The up-regulation will counteract this negative effect of deuteration. Albeit such a connection between the deuterium KIE and the cellular response may have been expected in retrospect, no experimental data at the system level had been presented before. Further, notably affected proteins comprise membrane transporters and proteins involved in nucleic acid processing and homeostasis, which may be related to variations of membrane mechanical properties and the overall stability of nucleic acids and proteins under deuterated conditions.

The observed changes of the proteome occur within several hours, which is faster than a possible evolution of the genome. Additional insights into this regulation of the proteome could be obtained by transcriptomics, whereas the affected enzymatic
functions may be further assessed by metabolomics. Conversely, selection of fast-growing *E. coli* clones over a very large number of generations in a deuterated medium should lead to persistent changes in the genome and epigenome. It will be interesting to compare such genetic changes induced by directed evolution to the observed short-term adaption of the proteome.

Because other prokaryotes, yeast, and algae can also be adapted to perdeuterated media, identical approaches can be used to reveal the most conserved features of the deuteration response. For higher eukaryotes, including the biotechnologically important insect and mammalian cells, analogous experiments may be carried out up to their tolerated deuteration maximum of ~30–50% (1, 4, 50, 51). This may in particular identify the crucial factors which lead to the described arrest of the cell cycle during mitosis (4, 32–34, 51).

In summary, we have shown that systems biology approaches as exemplified by proteomics reveal highly significant changes in *E. coli* under deuteration. This should provide a starting point for studying fundamental determinants of kinetics and stability in biomolecular systems via the deuterium isotope effect as well as for the development of improved biotechnological deuteration methods.

**Materials and methods**

**Growth media**

Lysogeny broth (LB) medium was prepared from 5 g of yeast extract (BD Biosciences), 10 g tryptone (BD Biosciences), and 10 g NaCl dissolved in 1 liter of deionized water and sterilized by autoclaving. For the preparation of LB agar plates, 20 g of agar (BD Biosciences) were added to 1 liter of LB medium before autoclaving. Protonated M9 minimal medium without a carbon source was prepared by mixing 700 ml of deionized H2O with 100 ml M9 salt solution (10×: 67.8 g/liter Na2HPO4, 30 g/liter KH2PO4, 5 g/liter NaCl in deionized H2O), 1 g NH4Cl, 10 ml of trace elements solution (100×: 5 g/liter EDTA, 0.5 g/liter FeCl3, 84 mg/liter ZnCl2, 10.3 mg/liter CuCl2, 0.86 mg/liter MnCl2, 5.5 mg/liter CoCl2 in deionized H2O), 0.3 ml 0.1 M CaCl2, 1 ml 1 M MgSO4, and 10 ml minimum Eagle’s medium vitamin solution (100×). Finally, 20 ml 20% (w/v) glucose solution were added and the resulting solution was filled up to 1 liter with deionized H2O. To prepare fractionally deuterated (70% D) M9 medium, 700 ml of this added H2O were replaced by D2O (99.8 atom % D). Deuterated (~99.8% D) M9 medium was prepared accordingly in 99.8% D2O using stock solutions prepared from anhydrous powders in D2O (99.8 atom % D) and 4 g/liter of glucose-1,2,3,4,5,6,6′-d7 (97 atom % D). All growth media were filter-sterilized before use. Unless indicated otherwise, all chemicals were obtained from Sigma-Aldrich.

**Growth and adaptation of *E. coli***

Cultures of *E. coli* BL21 (DE3) cells (genotype: F−, ompT, gal, dcm, lon, hisdSB(rB− mB−), λ(lacI, lacIII5-T7 gene 1, ind1, sam7, nin5)) (52) were grown on a rotary shaker (220 rpm, 50 mm throw) at 37 °C in batches of 15 ml in 100-ml Erlenmeyer flasks to provide sufficient aeration. First, cultures grown to stationary phase in LB medium were used to inoculate protonated M9 medium at an A600 of 0.01. Subsequently, cells in M9 medium were grown for five generations (~5.2 h total, M9H1). Adaptation to deuterated medium was achieved by repeated subculturing. For this, M9H1 cells were separated from the supernatant by centrifugation at 5000 × g for 5 min and resuspended in (70% D) M9 medium at A600 of 0.01 to grow for five generations (~7.3 h total, M9D2). Finally, the M9D2 cultures were again centrifuged and transferred to (~99.8% D) M9 medium at A600 of 0.01 to grow for five generations (~9.3 h total, M9D3). To ensure comparability of cells grown in protonated M9 medium to the M9D3 cells, M9H1 cultures were further subcultured twice in fresh M9 medium, yielding a total number of 15 generations (M9H3).

**Light microscopy and image analysis**

Samples were prepared for phase contrast imaging by adsorbing 3 µl of culture on a thin LB agar patch (5 × 5 mm) placed on a microscopic slide, briefly dried, and protected by a coverslip. Phase images were acquired on a DeltaVision imaging system (GE Healthcare) equipped with an Olympus IX71 microscope, a CoolSNAP HQ2 CCD camera (Photometrics), and a 100×/1.4 NA Ph3 Plan-Apochromat oil immersion objective (Olympus). Z stacks were collected 0.2 µm apart to cover the full volume of the bacteria. Image feature classification was performed using ilastik (53). Subsequently, cell size parameters were calculated from the ilastik output by the CellProfiler software package (54).

**EM**

Samples were prepared for cryo-TEM by adsorbing 4 µl of the undiluted culture suspension onto lacey carbon film mounted on 300 mesh copper grids (Ted Pella, Inc., Redding, CA). Prior to adsorption, the grid was rendered hydrophilic by glow discharge for 15 s. The specimen was applied to the grid surface and after 3-min incubation the grid was blotted en face and quick-frozen in liquid ethane using a Leica EM GP automated plunging device (Leica Microsystems, Vienna, Austria). The frozen grids were transferred under liquid nitrogen and loaded into a Gatan 626 cryo-holder (Gatan, Pleasanton, CA), which was then inserted into the stage of a FEI Talos transmission electron microscope (FEI Company, Hillsboro, OR) operated at 200 kV. Imaging was performed at cryogenic temperatures (approximately −170 °C) in low-dose, bright-field mode. Electron micrographs were recorded digitally on a CETA 16 M 4k × 4k CMOS Camera (FEI Company, Hillsboro, OR) at nominal defocus values of approximately −5 to −7 µm. The electron dose for low-dose image acquisition was maintained at ~30 e/Å2 per second.

**Whole-cell lysis and tryptic digestion of *E. coli***

Samples for proteome analyses were collected from the cultures grown for five generations in LB medium, protonated M9 medium (M9H1), and deuterated M9 medium (M9D3) as well as 15 generations in protonated M9 medium (M9H3) by centrifugation at 20,000 × g (4 °C) for 5 min. The obtained cell pellets were flash-frozen in liquid nitrogen and stored at −80 °C until further processing. Cells were resuspended in 100 mM ammonium carbonate containing 2% sodium deoxycholate (SDC) and 5 mM tris(2-carboxyethyl)phosphine (TCEP) lysis...
buffer). The cells were lysed by combined harsh vortexing (3 × 30 s) and sonication (3 × 10 s, 0.5 duty cycle, and 100% amplitude) using a VialTweeter (Hielscher). Obtained lysates were heated to 95 °C for 10 min while shaking at 500 rpm, followed by centrifugation at 10,000 × g for 10 s to remove cell debris. The protein concentration in the supernatant was determined using a BCA assay kit (Thermo Fisher Scientific). Subsequently, samples were diluted in lysis buffer to yield a protein concentration of 5 μg/μl and alkylation was performed on 100 μg of total protein in a volume of 20 μl using 10 mM of freshly prepared iodoacetamide for 30 min at 25 °C in the dark. The excess of iodoacetamide was quenched by addition of 12 mM N-acetylcyesteine.

For digestion, samples were diluted to yield a final SDC concentration of 1% using 100 mM ammonium carbonate. Subsequently, trypsin (Sigma-Aldrich) was added in a 1:50 enzyme/protein ratio (w/w) and digestion was performed overnight at 37 °C. The reaction was terminated by addition of 1% TFA, followed by centrifugation to remove SDC precipitate at 15,000 × g for 10 min (4 °C). Centrifugation was repeated once more and the obtained supernatant was used for solid phase extraction.

Peptides were desalted on a C18 reversed-phase spin column according to the supplier’s manual (Macrospin, Harvard Apparatus). All samples were prepared in biological triplicates.

**LC-MS/MS analysis**

To achieve reliable relative quantification of protonated and deuterated E. coli peptide mixtures, they were mixed 1:1, based on their total peptide content determined by a Bradford assay, into a single sample for LC-MS/MS analysis. In detail, exponential (ε) and stationary (s) peptide mixtures of each protonated condition (LBe/s, M9H1e/s, and M9H3e/s) were combined with the corresponding deuterated M9D3(e/s) peptide mixtures generating a total of 18 (9 for each growth phase) mixed MS samples.

Each sample was then subjected to LC-MS analysis using a dual pressure LTQ-Orbitrap Elite mass spectrometer connected to an electrospray ion source (Thermo Fisher Scientific) as described (55) with a few modifications. In brief, peptide separation was carried out using an EASY Scientific) as described (55) with a few modifications. In connected to an electrospray ion source (Thermo Fisher a dual pressure LTQ-Orbitrap Elite mass spectrometer MS samples.

**Identification and label-free quantification of protonated proteins**

For identification of the protonated peptides, MS raw files of all runs were imported into the Progenesis QI LC-MS software (Nonlinear Dynamics, version 2.0), aligned globally, and ana-
lyzed simultaneously using the default parameter settings. MS/MS data were then exported directly from Progenesis in MGF format and searched using Mascot (Matrix Science, version 2.4.0) against a concatenated target-decoy E. coli protein database including forward and reversed sequences (Uniprot, release date January 1, 2015, including 4479 target sequences). The search criteria were set as follows: 10 ppm precursor ion mass tolerance, 0.6-Da fragment ion mass tolerance, and full tryptic specificity required (cleavage after lysine or arginine resi-
dues); maximum three missed cleavages; fixed modification: carbamidomethylation (C), variable modification: oxidation (M). The database search results were filtered limiting the pep-
tide and protein level false discovery rate (FDR) to 1%. The Mascot peptide identifications were subsequently imported into Progenesis and mapped to the detected MS1 peaks to ≤90%. The assigned MS1 peptide intensities were further pro-
cessed using our in-house software script SafeQuant (55). This processing included normalization relative to the sum of all assigned MS1 peak intensities for individual LC-MS/MS runs and subsequent aggregation to protein abundances.

**Quantification of deuterated proteins**

An algorithm to analyze simultaneously both protonated and deuterated peptides within a single, mixed deuterated/proton-
ated MS sample was developed using Python. The algorithm uses the protonated peptide positions identified by Progenesis as a starting point to search and quantitate both protonated and deuterated peptides within the two-dimensional retention time, m/z LC-MS data matrix. At first, the position and intensity of the mono-isotopic protonated peptide is redetermined by a local-maximum pick peak picker within a relative m/z tolerance of 3 ppm and the global retention time window given by Pro-
genesis. The protonated peptide intensities determined by this algorithm correlated very well ($r^2 = 0.934, n = 4189$) with the corresponding Progenesis intensities (Fig. S3A). The correla-
tion between the peptide intensities translated into an even stronger correlation between the protein intensities ($r^2 = 0.959, n = 1772$). Minor deviations are caused by using raw intensity data, not yet filtered for assigned proteins, differences in the sample alignment and peak detection of the two approaches, as well as the fact that only the modification by carbamidomethylation was taken into account in the Python routine.

Subsequently, the algorithm searches the corresponding heavy peptides within the same relative mass tolerance of 3 ppm and a shifted retention time window, which takes into account that the retention times of deuterated peptides are reduced because of their weaker RP-HPLC matrix interaction. The shifted window of the deuterated retention times $RT_d$ was defined as $RT_p - \Delta t \leq RT_d < RT_p$, where $RT_p$ is the protonated retention time and $\Delta t$ defines the width of the window. The latter was determined using an iterative approach and set to 4
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min based on the mean retention time shift of the deuterated relative to the protonated peptides (Fig. S4). Within this window, the five most abundant isotopologue peaks matching the predicted m/z values were identified by a local maximum search. These five peaks typically present ~99.5% of the theoretical isotopologue pattern, which was calculated using a binomial distribution and a conservative estimate of the residual protonation level of 1.5% based on deuterium labeling of individual medium components. The peak of the fully deuterated isotopologue typically had the highest intensity of all five and was subsequently used for quantification.

The heavy peptide intensities determined by our algorithm showed a high linear correlation ($r^2 = 0.825, n = 4189$) to the corresponding light intensities (Fig. S3B) translating again into an even higher correlation of the respective protein intensities ($r^2 = 0.859, n = 1772$). The fact that the majority of peptide abundances remained unchanged under deuteration agrees with the expectation that the change in the deuterated proteome is minor and validates the reliability of the deuterated peptide identification by the algorithm. As a negative control for the algorithm, the retention time window for the heavy peptide identification was shifted by 9 min, while retaining the same absolute window size (Fig. S3C). No correlation was obtained between the light and heavy peptide intensities ($r^2 = 0.023, n = 3965$). A further negative control on MS data acquired on a protonated peptide sample without admixture of deuterated peptides also yielded no correlation ($r^2 = 0.010, n = 1540$).

The linearity of protein abundance detection using this approach was assessed by analyzing 1:0.1, 1:0.33, 1:1, 0.33:1, and 0:1:1 mixtures of 0.5 μg/μl protonated and 0.5 μg/μl deuterated E. coli peptide solutions. MS1 intensities of all commonly identified protonated and deuterated peptides ($n = 4104$) from three LC-MS/MS analyses were summed separately and their ratio was compared with the ratio of protonated and deuterated input peptide concentrations (Fig. S4). The data followed a linear dependence as MS(D)/MS(H) = 0.475 [D]/[H] ($r^2 = 0.9999$), where MS(D,H) presents the summed MS intensities of deuterated and protonated peptides, respectively, and [D]/[H] is the ratio of protonated and deuterated input peptide concentrations. The deviation of the proportionality factor 0.475 from unity is caused by the fact that only the highest intensity deuterated isotopologue peak was used in the summation, including the second highest intensity peak increases the proportionality factor to 0.898.

Following the identification and quantification of the individual peptides, the algorithm then combines the median values of the three most intense protonated and deuterated peptide intensities for a given protein to obtain total protein abundances for each condition and replicates thereof. The respective Python scripts are available upon request.

Analysis of the relative change in the protein abundance

To test for differential abundance, the empirical Bayes method (56) was applied and the obtained $p$ values, which reflect the probability of detecting a given mean abundance difference across sample conditions by chance alone were corrected for multiple testing ($q$-values) using the Benjamini-Hochberg method (57).

For each condition, individual errors in the intensities were obtained from the standard deviation of triple biological replicates. A minimal error was imposed as 25% of the median of all intensity standard deviations of these replicates. The final errors in the relative protein abundances used for the COGs and GO analyses were derived by error propagation.

Clusters of orthologous groups of proteins annotations

COGs classifications were obtained from the COGs database (www.ncbi.nlm.nih.gov/COG, updated 2014).

Statistical analysis of Gene Ontology classes

Gene product annotations were obtained from the GO consortium database filtered for E. coli (www.geneontology.org, release date May 24, 2017, including 3545 genes) (59, 60). The enrichment analysis was constrained to GO classes for which at least 75% of the associated protein abundances were detected by LC/MS. Proteins within these classes were categorized into strongly (at least 2-fold) up- or down-regulated and into not strongly (less than 2-fold) regulated based on their experimental variation. The statistical analysis was performed using Python 2.7.

Accession codes

All MS raw data files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (58) with the data set identifier PXD009736.

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