Sulfur-34S Stable Isotope Labeling of Amino Acids for Quantification (SULAQ34) of Proteomic Changes in Pseudomonas fluorescens during Naphthalene Degradation*

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The relative quantification of proteins is one of the major techniques used to elucidate physiological reactions. Because it allows one to avoid artifacts due to chemical labeling, the metabolic introduction of heavy isotopes into proteins and peptides is the preferred method for relative quantification. For eukaryotic cells, stable isotope labeling by amino acids in cell culture (SILAC) has become the gold standard and can be readily applied in a vast number of scenarios. In the microbial realm, with its highly versatile metabolic capabilities, SILAC is often not feasible, and the use of other 13C or 15N labeled substrates might not be practical. Here, the incorporation of heavy sulfur isotopes is shown to be a useful alternative. We introduce 34S stable isotope labeling of amino acids for quantification and the corresponding tools required for spectra extraction and disintegration of the isotopic overlaps caused by the small mass shift. As proof of principle, we investigated the proteomic changes related to naphthalene degradation in P. fluorescens ATCC 17483 and uncovered a specific oxidative-stress-like response. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.025627, 2060–2069, 2013.

Proteomics relies on accurate protein quantifications to provide comprehensive insight into physiological changes. In the past two decades, a vast variety of quantification methods based on mass spectrometry (MS) analysis have been developed and reviewed (1). One of the favored principles is the metabolic introduction of stable isotopes into proteins by means of growth on correspondingly labeled substrates. MS analysis allows detection of the resulting mass shift and thus the differentiation of signals from unlabeled (light) and labeled (heavy) analytes. When labeled and unlabeled samples are combined, a relative quantification based on the comparison of the signal intensities is possible. A specific approach that has become important in quantitative proteomics is stable isotope labeling by amino acids in cell culture (SILAC)1 (2), in which stable-isotope-labeled amino acids (e.g. 13C and/or 15N labeled lysine and arginine) are added to the medium. Although SILAC is a powerful method for investigating eukaryotic cell cultures that depend on amino-acid-supplemented media, the method so far is applicable to only a few prokaryotes such as Staphylococcus aureus (3) or Bacillus subtilis (4). The metabolization or self-synthesis of amino acids, as often occurs in microbes, can lead to an unpredictable spread of the label. Even in auxotrophic and pseudo-auxotrophic cell cultures or microorganisms, it cannot be ruled out that the addition of amino acids itself might influence the proteome. Therefore, in microbiology, the direct labeling of carbon or nitrogen sources is a more suitable strategy. The feasibility of this approach for protein quantification has been demonstrated (5), but the required C and N substrates might be too expensive or rarely available, or the experimental setup might involve changes in carbon and nitrogen sources that make labels thereof unfeasible. An alternative element present in proteins and essential for microbial growth is sulfur. Sulfur, which is usually supplemented as sulfate in bacterial growth media, is present in only two amino acids, methionine and cysteine. This leads to very defined mass shifts when heavy isotopes replace 32S. Although the overall protein quantifica-

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1 The abbreviations used are: LFQ, label-free quantification; PAH, polycyclic aromatic hydrocarbon; SILAC, stable isotope labeling by amino acids in cell culture; SULAQ, sulfur stable isotope labeling of amino acids for quantification.
tion coverage will probably be less than that obtained with nitrogen or carbon labeling, sulfur’s universal presence in proteins can be helpful in experiments when carbon or nitrogen labeling is neither possible nor economically feasible. Furthermore, in experiments in which specifically defined C- or N-substrates cannot be given, sulfur labeling would be an excellent alternative. Thus metabolic labeling using stable sulfur isotopes can be a helpful addition to the repertoire of quantitative proteomics. Jehmlich et al. (6) compared the proteome of P. putida with either sodium benzoate or sodium succinate as a carbon source in a quantification approach with $^{36}$S-sulfate. They proved that the $^{36}$S-sulfate did not interfere with growth behavior or chromatographic retention times and is therefore usable as stable isotopic marker. The drawback of $^{36}$S-sulfate is that it is commercially available only as elemental $^{36}$S-sulfur and has a high cost. This leads to the need for the additional synthesis of $^{36}$S-sulfate, which contributes further to its high price.

In this article we introduce $^{34}$S as a new metabolic stable isotope marker for bacteria with a cost a tenth of that of $^{36}$S. A further advantage is its ready commercial availability as sodium sulfate. The power of $^{34}$S in proteomics has already been demonstrated (7, 8), but it has not been utilized as a means for routine protein quantifications. When only one sulfur atom is replaced, the 2 Da mass shift is not large enough to completely separate the isotopic clusters of light and heavy labeled peptides, leading to isotopic overlap and thus hampering reliable quantification. Because the mass shift caused by the replacement of two $^{12}$C atoms with two $^{13}$C atoms and that caused the replacement of one $^{32}$S atom with its $^{34}$S isotope differ by only 0.011 Da, a high mass resolution is required in order to distinguish between these two types of incorporation (9). This is rarely available in routine peptide MS. Therefore, we have developed a tool that can be used to disentangle the isotopic patterns and make $^{34}$S labeling widely applicable.

In order to demonstrate the feasibility of $^{34}$S labels for protein quantification, the proteome of Pseudomonas fluorescens ATCC 17483 was investigated. Pseudomonas species are able to degrade a high number of (xenobiotic) compounds (10), making them very interesting for bioremediation processes (11, 12). Using SILAC, the investigation of such self-sufficient organisms is not possible. One typical group of important environmental contaminants degraded by Pseudomonads is the polycyclic aromatic hydrocarbon (PAH) group. PAHs consist of two or more benzene rings in linear, angular, or cluster arrangements (13). It is assumed that they are toxic, mutagenic, and even carcinogenic. Because their hydrophobic properties result in low bioavailability, they tend to be highly persistent in the environment, exhibiting potential hazardous effects. Because of their abundance in crude oil and their widespread use in chemical manufacturing, PAHs are widely distributed in nature. Although PAHs’ degradation pathways—especially those of low-molecular-weight PAHs (13)—have been studied in detail in Pseudomonades, no transcriptomic or global proteomic study has been performed.

In this proteomics study utilizing $^{34}$S stable isotope labeling of amino acids for quantification (SULAQ34), naphthalene was used as a model low-molecular-weight PAH to investigate the PAH metabolism in P. fluorescens and thus enhance our understanding with regard to physiological adaptation.

**EXPERIMENTAL PROCEDURES**

**Cultivation—**Preparatory cultures of P. fluorescens ATCC 17483 were grown in 5 ml mineral medium (760 mg/l NH₄Cl, 680 mg/l K₂PO₄, 871 mg/l KH₂PO₄, 5.5 mg/l CaCl₂×6H₂O, 0.25 mg/l Na₂MoO₄×4H₂O, pH 7.0) supplemented with trace element solution SL-10 (1 ml/l)(14, 15), MgCl₂×6H₂O (1 mX), and sodium succinate (10 mM) as the carbon source. The sulfur source was either $^{32}$S- or $^{34}$S-sulfate (2 mM) (99.9%, Campro Scientific, Berlin, Germany). Overnight grown pre-cultures were used to inoculate main cultures with either sodium succinate (10 mM) or crystalline naphthalene (0.1% w/v) as the carbon source according to their prior sulfur source (see Fig. 1). Because of an extended lag phase during PAH exposure, naphthalene cultures were inoculated approximately 12 hours earlier than the sodium succinate cultures. Cells of $^{32}$S and $^{34}$S cultivations were subsequently harvested during the early exponential phase ($A_{600}$ = 0.25, approximately six generations) and mixed 1:1 (based on OD, –2 ml each). Combined cell pellets were obtained via centrifugation (12,000 rpm, 4 °C, 20 min). Overall, eight cultivations were performed. The cultures were $^{32}$S-SQ1 to $^{32}$S-SQ4 and $^{34}$S-SQ1 to $^{34}$S-SQ4. Naphthalene was the sole carbon source in $^{32}$S-SQ1/32S-SQ2 and in $^{34}$S-SQ3/34S-SQ4, and succinate was the sole carbon source in the cultures $^{32}$S-SQ3/34S-SQ4 and $^{34}$S-SQ1/34S-SQ2 (see Fig. 1). Overall, eight cultivations (four for each substrate) including a label switch resulted in four $^{32}$S/$^{34}$S combined biological replicates, numbered SQ1 to SQ4, for quantification. Additionally, 1 ml from each $^{32}$S culture was harvested and measured separately for label-free supportive studies.

**Sample Preparation—**Cell pellets were suspended in 30 µl SDS sample buffer and heated to 95 °C for 15 min. Afterward, suspensions were centrifuged (16,000 rpm, 5 min, ambient temperature) and used for SDS-PAGE. Gels were cut into ten pieces, and in-gel digestion was carried out. Descriptions of the SDS sample buffer, SDS-PAGE adjacent clean-up steps, and in-gel digestion can be found in Ref. 16. Eluted peptides were purified and concentrated using C18 Zip Tip columns (Millipore Bellerica, MA) (see Fig. 1).

**Mass Spectrometry—**Peptides were reconstituted in 0.1% formic acid for LTQ-Orbitrap MS analysis. Samples were injected by the autosampler and concentrated on a trapping column (nanoAcquity UPLC column, C18, 180 µm × 2 cm, 5 µm, Waters Milford, MA) with water containing 0.1% formic acid at flow rates of 15 µl min⁻¹. After 6 min, the peptides were eluted into a separation column (nanoAcquity UPLC column, C18, 75 µm × 15 cm, 1.75 µm, Waters). Chromatography was performed with 0.1% formic acid in solvents A (100% water) and B (100% acetonitrile). Using a nano-high pressure liquid chromatography system (nanoAcquity UPLC, Waters) coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific), the SULAQ34 peptides were eluted. The solvent B gradient was set to increase from 2% to 15% for the first 10 min and from 15% to 40% for the subsequent 67 min, with a final switch to 85% solvent B for an additional 10 min. The label-free quantification (LFQ) elution gradient was set analog to the prior described elution gradient. For an unbiased analysis, continuous scanning of eluted peptide ions was carried out between 300 and 2000 m/z, automatically switching to MS/MS collision-induced dissociation mode on ions exceeding an
The intensity of 3000, and with six MS/MS events per survey scan. For MS/MS collision-induced dissociation measurements, a dynamic precursor exclusion of 3 min was enabled.

**Data Analysis**—Because a complete genome sequence of the specific strain ATCC 17483 is not yet available, protein entries of all *P. fluorescens* strains (taxonomy ID: 294, 25,967 entries) that were available at the UniProt website on January 9, 2012, were used for protein identification. Identification was performed with MaxQuant (v. 1.2.2.5) (17) and its built-in database search algorithm Andromeda (18). In addition to the standard settings, $^{34}$S replacement in methionine and cysteine was added as a variable modification, and activation of the match-between-runs feature with a 2 min alignment window was used (see supplemental Dataset1.xlsx for an overview of all applied settings). Proteins needed to be identified with at least two unique peptides. The protein and peptide false discovery rate limits were set at 1%. In the case of the label-free supportive study, the LFQ feature was enabled as well (see supplemental Dataset2.xlsx).

**SULAQ34 Quantification**—The quantification pipeline consisted of several steps (see Fig. 1 and supplemental SULAQ34_HowTo.pdf). First, raw files were converted to mzMLs by msconvert from the ProteoWizard suite (19) (release 2.1.2465). Besides activated peak picking, default settings were used for the conversions. In the next step, PeakExtractor (see supplemental PeakExtractor.zip) was used to extract all MS1 spectra belonging to one peptide as defined in a list containing peptide identification information comprising the peptide sequence, peptide ID, mass, retention time or scan number, and charge. Elution profiles were elucidated using the provided peptide mass within an error tolerance of 10 ppm and one scan gap. Averaging of extracted spectra was done using the Kroénik algorithm (20). Kroénik is able to condense spectra information to averaged spectra with specifiable tolerances and a minimum number of spectra per mass signal. A visual basic for applications macro extracted all mass signals belonging to the peptide of interest and transferred these to the 34Scalc Excel script (see supplemental 34Scalc.xls). The Excel script enables easy disintegration of overlapping isotopic patterns of $^{32}$S/$^{34}$S origin (see Fig. 2), along with automatic quantification based on spectral data supplied in a form as shown in the example sheet in supplemental 34Scalc.xls. $^{32}$S/$^{34}$S peptide ratios provided by the

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**FIG. 1.** Experimental setup for cultivation and label switch, as well as the downstream IT pipeline for protein quantification. The $^{32}$S and $^{34}$S captions represent the applied label; “Succ” and “Naph” are the carbon sources succinate and naphthalene. Overall, eight cultivations led to four biological replicates: SQ1, SQ2 ($^{32}$S-naphthalene + $^{34}$S-succinate) and SQ3, SQ4 ($^{32}$S-succinate + $^{34}$S-naphthalene).

**FIG. 2.** Schematic workflow of *in silico* disintegration of overlapping isotopic patterns.
RESULTS AND DISCUSSION

Identification and Quantification Numbers—Proteins were identified by means of mass spectrometric peptide analysis and quantified based on peptide signal intensity. In total, 886 proteins were identified. For protein quantification, log2 values based on peptide intensity ratios were calculated. All relative quantification values refer to the situation during naphthalene utilization in which positive log2 ratios indicate higher abundance relative to succinate utilization, and vice versa. Overall, 387 proteins were quantified that were identified in at least two replicates. Of these, 88 appeared to be more abundant (log2 ratio > 0.8 or on, median p value < 0.05) and 42 appeared to be less abundant (log2 ratio < -0.8 or off, median p value < 0.05) during naphthalene metabolism (see Fig. 3). The whole list of quantified proteins, including replicate values, standard deviations, and median quantifications, can be found in the supplemental material.

In all, 717 proteins were identified and quantified in the LFQ experiments. Of these, 45 were found solely in the sodium succinate samples, and 75 proteins were observed only in the naphthalene samples. Another 138 proteins were found to be more abundant during naphthalene metabolism, and 156 to be less abundant (see the supplemental material for a complete list).

SULAQ34—The use of 34S-sulfate in culture media leads to a high labeling efficiency (see Fig. 4) and distinct isotopic patterns, which make bioinformatic evaluation easier. To demonstrate the suitability of SULAQ34 for protein quantification, calculated log2 values were averaged at the protein level and checked for consistency among replicates. Standard deviations of the replicate log2 values for each protein were calculated, showing a mean of 0.48, which indicated sufficient stability between replicates. The median of the standard deviations was 0.38. The higher value of the mean was caused by a few extreme outliers (see Fig. 5). The same is true for the median of the coefficients of variation of the fold changes, which was as much as 25% (mean = 31%). The deviations between the replicates can be explained by biological variations among the cultures. The label switch itself did not introduce a visible systematic bias (see Fig. 5). A further possible source of errors is the fact that this specific organism is not sequenced, and peptides might not always be assigned to the correct protein, especially when similar gene copies are present. For example, different peptides were assigned to two versions of DnaJ, Q4KIH0 and C3K274, leading to two different quantifications. These quantifications differed significantly but were consistent among replicates, indicating that two distinct proteins exist, potentially with differing functions. Of the 130 proteins determined to be significantly more or less abundant in the SULAQ approach, 109 were also quantified in the LFQ experiment. Of these, 65% were deemed up- or down-regulated in the SULAQ34 and in the LFQ experiment. 31% were recognized as significantly altered in abundance solely in the SULAQ34 samples. In less than 4% of the cases, the SULAQ34 and LFQ experiments gave con-
tradictory indications. Thus, data were overall in good agreement between SULAQ34 and LFQ experiments.

General Physiological Observations—The shift from sodium succinate to naphthalene as the carbon and energy source had a strong effect on physiology. One-third of all quantified proteins underwent significant changes in abundance (130 of 387; see Fig. 3), showing the importance of global approaches in understanding microbial processes. The substrate shift led to a significantly lower abundance of Q4KDB8, a transporter solute receptor of the DctP family constituting a part of the tripartite ATP-independent periplasmic transport system (see Table I for a list of discussed proteins). The tripartite ATP-independent periplasmic transport system and, especially, solute receptors of the DctP family are important in the uptake of C4-dicarboxylates like succinate (21). Further changes were mainly related to proteins with yet unknown functions such as the uncharacterized protein kinase YeaG (22) or the cold shock protein CspA, which seems to be of general importance during aromatic hydrocarbon exposition in Pseudomonades (23). Furthermore, the changes involved general aspects like amino acid and carbohydrate metabolism, transport or regulation, and signal transduction, as well as enzymes involved in PAH degradation and a specific stress response that is discussed in more detail in a later section. For a complete list of proteins and quantification values, see supplemental SI_Quantification.xlsx.

Naphthalene Metabolism—We could identify and quantify 14 out of 15 enzymes responsible for the degradation of naphthalene to its two major end products, pyruvate and acetyl-CoA. All 14 enzymes were found in significantly higher abundances or regarded as on proteins in naphthalene cultures. This was to be expected, as PAHs metabolizing enzymes are tightly controlled and expressed significantly only in the presence of naphthalene or salicylate in P. fluorescens ATCC 17483 (24, 25). Even though we anticipated this outcome, it empirically proves the feasibility and correctness of the applied quantification methodology. Possible entry points for acetyl-CoA and pyruvate into central metabolic pathways are the tricarboxylic acid cycle and glycolysis. Pyruvate could mainly be used by the phosphoenolpyruvate synthase Q4K5J5, which also showed an increase in abundance, to supply phosphoenolpyruvate carboxykinase with phosphoenolpyruvate forming oxaloacetate. Oxaloacetate and acetyl-CoA could then be utilized by citrate synthase to form citrate. Citrate synthase, which provides the final access for naphthalene degradation metabolites to the central metabolism, was found to be more than twice as abundant. Furthermore, in our experiment two alcohol dehydrogenases (26) were found to be regulated opposite to each other. ExaA, a quinoprotein ethanol dehydrogenase, was 30-fold less abundant, whereas YahK, belonging to the zinc-containing alcohol dehydrogenase superfamily, was 10-fold more abundant. YahK contains a cinnamyl alcohol dehydrogenase domain. The role of cinnamyl alcohol dehydrogenase–like alcohol dehydrogenases in bacteria is still largely unclear (26), and YahK might play a role in the metabolization of aromatic alcohols (27). Further investigation of these enzymes might lead to the discovery of side products, pathways yet unknown, or possible moonlight functions of proteins such as FliC, which was detected at a higher abundance. Usually FliC is an essential part of the flagellum, but we did not find evidence of a strengthened flagellar system. However, FliC has already been reported to be noticeable in, for instance, hyperosmolaric conditions (28).

Naphthalene-promoted Stress—Our results show that several proteins of the microbial stress response were found at
**TABLE I**

Selection of regulated proteins involved in PAH degradation and stress response

| Cluster | Accession | SO1 | SO2 | SO3 | SO4 | Tendency | Average log2 | LFQ log2 | Description |
|---------|-----------|-----|-----|-----|-----|----------|-------------|----------|-------------|
| PAH degradation | Q938R2 | On | On | On | On | ↑↑ NA | On | On | Ferredoxin reductase GN – nahAa |
|          | Q938S0 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dioxygenase GN – nahAb |
|          | Q938R9 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dioxygenase GN – nahAc |
|          | C07825 | On | On | On | On | ↑↑ NA | On | On | Naphthalene 1,2-dioxygenase subunit beta (fragment) GN – ndoC |
|          | Q938R1 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dehydrogenase GN – nahB |
|          | Q8KP17 | On | On | On | On | ↑↑ NA | On | On | 1,2-dihydroxynaphthalene dioxygenase GN – nahC |
|          | C3KFM7 | On | On | On | On | ↑↑ NA | On | On | 1,2-dihydroxynaphthalene dioxygenase GN – nahC |
|          | Q938R8 | On | On | On | On | ↑↑ NA | On | On | Ferredoxin reductase GN – nahAa |
|          | Q93CM5 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dioxygenase GN – nahAb |
|          | Q938R0 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dioxygenase GN – nahAc |
|          | C79P83 | On | On | On | On | ↑↑ NA | On | On | Naphthalene 1,2-dioxygenase subunit beta (fragment) GN – ndoC |
|          | Q52VQ6 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dehydrogenase GN – nahB |
|          | Q8KRR9 | On | On | On | On | ↑↑ NA | On | On | 1,2-dihydroxynaphthalene dioxygenase GN – nahC |
|          | C3KFP9 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dioxygenase GN – nahB |
|          | Q8KRR6 | On | On | On | On | ↑↑ NA | On | On | 1,2-dihydroxynaphthalene dioxygenase GN – nahC |
|          | Q8KRR7 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dioxygenase GN – nahB |
|          | Q7B5H5 | On | On | On | On | ↑↑ NA | On | On | 1,2-dihydroxynaphthalene dioxygenase GN – nahC |
|          | Q8KRR8 | On | On | On | On | ↑↑ NA | On | On | Naphthalene dioxygenase GN – nahB |
| Stress response | C3KA77 | On | On | 0.47 | ↑ NA | On | On | Glutathione reductase |
|            | Q3K7D8 | 1.93 | 2.61 | 2.47 | ↑↑ | 2.34 | 1.80 | ATP-dependent Pim1 peptidase; serine peptidase; MEROPS family S16 |
|            | C3KA80 | 2.73 | 2.17 | 1.39 | 2.12 | ↑↑ | 2.10 | 0.78 | Alkyl hydroperoxide reductase protein |
|            | E2XP36 | 1.69 | 1.54 | 2.68 | 1.53 | ↑↑ | 1.86 | 0.10 | Chaperone protein htpG GN – htpG |
|            | Q3KD92 | 1.73 | 1.31 | ↑ NA | 1.52 | | | Multidrug efflux system transmembrane protein |
|            | Q3KB6 | 2.31 | 0.90 | 1.11 | ↑ | 1.44 | | | ATP-dependent protease ATPase subunit HsiU GN – hsiU |
|            | C3K3J7 | 2.73 | 0.42 | 0.75 | 1.62 | ↑ | 1.38 | | | Glutathione synthetase GN – gshB |
|            | C3K2S6 | 2.73 | 1.82 | 0.50 | 0.45 | ↑ | 1.38 | 0.38 | Putative thioredoxin |
|            | Q4KIH1 | 1.81 | 1.44 | 1.07 | 1.14 | ↑↑ | 1.37 | 0.84 | Chaperone protein DnaK GN – dnaK |
|            | Q4K764 | 1.46 | 1.41 | 1.10 | 1.47 | ↑↑ | 1.36 | 0.84 | 60 kDa chaperonin GN – groL |
|            | Q3KAA1 | On | On | 1.17 | 1.45 | ↑ NA | 1.31 | 2.23 | Putative gamma-glutamyltranspeptidase GN – ggt |
|            | Q3KX1 | 0.76 | 1.22 | 1.16 | 1.47 | ↑↑ | 1.15 | 0.84 | ATP-dependent proteinase; MEROPS family S16 GN – lon |
|            | E2XTC4 | 1.86 | 0.72 | 0.64 | ↑ | 1.07 | 3.58 | PpcG-type peptidyl-prolyl cis-trans isomerase GN – ppcG |
|            | C3KB0 | On | 0.80 | 0.75 | 1.53 | ↑ | 1.02 | 0.29 | Peptidyl-prolyl cis-trans isomerase GN – fkb |
|            | Q4KF8 | On | 0.99 | 1.26 | 0.81 | ↑ | 1.02 | 1.08 | Chaperone protein htpG GN – htpG |
|            | Q3KX0 | 2.51 | –0.54 | 1.06 | 0.78 | ↑ | 0.95 | 0.55 | ATP-dependent Cip protease ATP-binding subunit CipX GN – cipX |
|            | Q3K6N3 | –1.53 | 0.83 | 1.96 | 2.15 | ↑ | 0.86 | On | Chaperone GN – cipB |
|            | E2XV7 | 1.51 | –0.42 | 1.17 | 1.13 | ↑ | 0.85 | 0.17 | Cold shock protein (beta-ribbon CspA family) GN – cspA |
|            | Q3KE2 | 1.15 | 0.54 | ↑ NA | 0.84 | 0.62 | | | Catalase-peroxidase GN – katG PE – 1 SV – 1 |
|            | C3K709 | –0.81 | –0.72 | –0.90 | ↓ | –0.81 | 1.57 | | | Cold shock protein GN – cspB PE – 3 SV – 1 |
|            | Q3K6M2 | –0.84 | –1.00 | 0.72 | ↓ | –0.86 | | | Cold-shock dead-box protein A GN – deaD PE – 3 SV – 1 |
|            | Q3K933 | –1.06 | –1.25 | –1.19 | –1.47 | ↓ | –1.24 | 1.46 | Protease HtpX GN – htpX |

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Multidrug efflux systems can serve to keep the cytosolic concentration at an acceptable level (31). Furthermore, a conspicuous number of proteases, chaperones, and two peptidyl-prolyl cis-trans isomerases (PPIases) were found at significantly altered levels, mainly at higher abundances. Given its hydrophobic nature and formation of toxic side-products, it is likely that naphthalene can lead to protein misfolding and aggregation. PPIases are known to counteract misfolding and are highly relevant in the promotion of stress tolerance (e.g. for oxidative stress) or in influencing membrane protein profiles and fatty acid composition in Gram-negative bacteria such as *Yersinia pseudotuberculosis* (32). Besides their importance for stress tolerance, they have been shown to be necessary for pilus biogenesis in *E. coli* (33) and might be of importance in many yet unknown processes (34).

In addition, ClpB and DnaK were found to be increased (−2.5-fold). It is known that ClpB and DnaK can function separately (35) or as a chaperone to disintegrate protein aggregates (36, 37). They have previously been found in higher abundances simultaneously during cold stress (38) and together with HtpG during oxidative stress (39, 40). Our increased abundances in naphthalene-grown cells. The identified alterations indicate a stress response similar to the known adaptation toward oxidative stress. How *P. fluorescens* deals with naphthalene-promoted stress is discussed in more detail in this paragraph. One mechanism by which bacteria overcome membrane stress caused by hydrophobic substances is alteration of their membrane lipid composition (29). Noteworthy is a putative glycerocephospholipid diester phosphodiesterase that might be important for membrane remodeling to cope with naphthalene stress. The biotin carboxylase AccC, which provides building blocks for lipid synthesis, was also significantly more abundant. Also, the higher abundant fatty acid oxidation complex might be of importance. In addition, evidence suggests a higher demand for branched chain amino acids, which might be needed for an increase of branched chain fatty acids in the membrane (see *supplemental SI_Quantification.xlsx*). This has previously been observed in mycobacteria (30). Q3KD92, a part of the multidrug efflux system, was also increased in abundance. Multidrug efflux systems can serve to keep the cytosolic naphthalene concentration at an acceptable level (31). Furthermore, a conspicuous number of proteases, chaperones, and two peptidyl-prolyl cis-trans isomerases (PPIases) were found at significantly altered levels, mainly at higher abundances. Given its hydrophobic nature and formation of toxic side-products, it is likely that naphthalene can lead to protein misfolding and aggregation. PPIases are known to counteract misfolding and are highly relevant in the promotion of stress tolerance (e.g. for oxidative stress) or in influencing membrane protein profiles and fatty acid composition in Gram-negative bacteria such as *Yersinia pseudotuberculosis* (32). Besides their importance for stress tolerance, they have been shown to be necessary for pilus biogenesis in *E. coli* (33) and might be of importance in many yet unknown processes (34).

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### Table I—continued

| Cluster | Accession | SQ1  | SQ2  | SQ3  | SQ4  | Tendency | Average log2 | LFQ log2 | Description |
|---------|-----------|------|------|------|------|----------|-------------|----------|-------------|
| Miscellaneous and general metabolism | E2XWP5 | On   | 3.19 | 3.19 | ↑ † ** | 3.19 | Zinc-containing alcohol dehydrogenase superfamily GN = yahJ |
|         | C3K4F7 | On   | 2.59 | 2.78 | ↑ † NA | 2.69 | 2.53 | Biotin carboxylase GN = accC PE = 4 SV = 1 |
|         | C7DRJ0 | −2.27 | 2.17 | 1.82 | 2.67 | ↑ NA | 1.10 | 2.62 | Aldehyde dehydrogenase |
|         | C3K1K9 | 2.32 | 1.63 | ↑ NA | 1.97 | 1.97 | 1.07 | Putative aminotransferase |
|         | E2XPE5 | On   | −0.50 | 1.69 | 1.60 | ↑ NA | 0.93 | 0.93 | Carboxyl-terminal processing protease |
|         | Q3KA61 | 0.81 | 1.15 | 1.73 | 1.56 | ↑ ** | 1.31 | 1.31 | NADH-quinone oxidoreductase subunit |
|         | Q3KG66 | 2.51 | 1.54 | 0.83 | 1.06 | ↑ † | 1.48 | 0.27 | Aromatic amino acid aminotransferase apoenzyme |
|         | Q4KG78 | 1.36 | 2.63 | 0.72 | 1.14 | ↑ † | 1.46 | 0.34 | Flagellin FliC GN = fliC |
|         | Q3K9F6 | 1.15 | 1.31 | ↑ NA | 1.23 | 1.23 | 1.23 | Putative glycerophosphoryl diester phosphodiesterase |
|         | Q4KH87 | 1.67 | 1.54 | 0.45 | 0.75 | ↑ † | 1.10 | 1.33 | Histidine ABC transporter, periplasmic histidine-binding protein HsiJ GN = hisJ |
|         | C3JY81 | 0.86 | 2.04 | 0.81 | 0.24 | ↑ † | 0.99 | Off | NADH-quinone oxidoreductase subunit |
|         | C3K673 | 0.99 | 1.03 | 1.22 | 1.31 | ↑ † | 1.14 | 1.15 | Citrate synthase GN = gltA |
|         | C3K9D8 | 0.92 | 1.31 | 1.07 | 1.17 | ↑ † | 1.12 | 0.89 | Fatty acid oxidation complex subunit |
|         | Q3KA59 | 2.32 | 1.72 | 0.47 | 0.29 | ↑ † | 1.20 | 0.10 | NADH dehydrogenase subunit F |
|         | Q3K908 | 1.51 | 2.17 | 0.68 | 0.65 | ↑ † | 1.25 | 0.93 | Phospho-2-dehydro-3-deoxyxanthopteridine aldolase GN = aroF |
|         | Q3K5U9 | On   | 1.11 | 1.09 | 0.88 | ↑ † | 1.03 | 1.03 | Anthranilate synthase component I |
|         | Q4KFJ5 | 0.97 | 0.95 | 0.74 | 0.89 | ↑ † | 0.89 | 0.46 | Pyruvate, water dikinase GN = ppsA |
|         | C3K1E6 | −1.23 | −0.88 | −0.84 | −0.73 | ↓ ** | −0.92 | −1.34 | ATP synthase subunit beta GN = atpD |
|         | Q4K3A7 | −1.13 | −1.26 | −0.72 | −0.86 | ↓ ** | −0.99 | −2.08 | ATP synthase subunit alpha GN = atpA |
|         | Q4KDB8 | −0.97 | −1.53 | −2.01 | ↓ ** | −1.50 | Off | Tripartite ATP-independent periplasmic transporter solute receptor, DctP family |
|         | Q4KX1  | −1.03 | −2.20 | −2.80 | −2.03 | ↑ † | −2.01 | −2.66 | Protein kinase YeaG GN = yeaG |
|         | E2XUN3 | −4.95 | Off | Off | Off | ↑ NA | −2.01 | −2.66 | Quinoprotein ethanol dehydrogenase GN = exaA |

On, greater than 20-fold increase in abundance with naphthalene; Off, greater than 20-fold decrease in abundance with naphthalene; *, p value < 0.1; **, p value < 0.01; NA, fewer than two values available for a t test.
results show that HtpG also was more abundant and, like DnaK, it can work in cooperation with ClpB (41). Usually DnaK acts together with DnaJ. As described above, it seems that two proteins showing similarity to DnaJ could not be discriminated and are regulated antagonistically. Misfolded proteins that cannot be rescued lead to severe consequences for bacteria. They are responsible for bacterial cell aging (42) and are assumed to be toxic (43). To prevent cell damage, it is essential to degrade these protein species. Among the proteins with increased abundance were the ATPase subunit HslU of HslU/HslV. Missiakas et al. were able to show that HslU/HslV is important for the degradation of misfolded proteins in E. coli (44), and recent studies identified HslU and ClpB as increased when confronted with higher inclusion body formation due to protein overexpression (45). Also, the carboxyl-terminal processing protease PrC was found in higher amounts. It has been associated with oxidative stress protection in fungi (46). Further, the Lon protease, which is known for the degradation of protein aggregates (47), was present at a higher abundance. The lower abundance of HtpX, a membrane-bound protease involved in quality control (48), suggests that a defined set of proteases, and not a general up-regulation of degradation, is part of the stress response to naphthalene exposure.

It seems that the overall stress control mechanism has to cope with higher protein misfolding. Reasons for this are usually heat stress, artificial protein overexpression, and/or oxidative stress. Heat stress or stress caused by protein overexpression cannot be the case here. As mentioned before, many proteins of the protein quality control system are essential for oxidative stress tolerance (32, 39). This is highlighted by the higher abundance of an alkyl hydroperoxide reductase, a putative thioredoxin, and glutathione synthase and reductase. Glutathione and thioredoxin are essential for protecting bacteria from oxidative damage (49, 50). Catalase, which is essential for hydrogen peroxide decomposition (51) and a general marker for oxidative stress (52), was slightly more abundant as well. The reason for only a slight increase might be that either catalase was replaced by an unrecognized alternative expressed strongly enough constitutively (53) or the oxidative agent did not trigger catalase synthesis, as the response to oxidative stress can be very dependent on the oxidative agent (54) or its level (55). In addition to direct countermeasures, metabolic adjustments can prevent the formation of additional reactive oxygen species. The alpha and beta subunits of an ATP synthase were less abundant, potentially implying lowered respiratory chain and proton translocation activity, which would lead to decreased reactive oxygen species formation. Contradictorily, several parts of the respiratory chain, namely, NuoA, NuoC, and NuoF, are more abundant, but lower ATP concentrations during oxidative stress have been reported (39). A high ratio of NADPH to NADH also helps in antioxidative protection. It can be expected that organisms are intrinsically adapted to cope very specifically with stress in their metabolism. It has previously been shown that the allocation of NADPH / H+ is one of the main metabolic adjustments in P. fluorescens to counteract oxidative stress (56). Several enzymes seem to be of importance for this, such as an almost 3-fold more abundant aldehyde dehydrogenase (C7DRJ0), which also might be part of the aldehyde detoxification during naphthalene degradation (57). The first evidence has been found that the kind of NADPH regeneration is directly influenced by the available carbon source (56). Our findings suggest that aldehyde dehydrogenase is of major importance for NADPH allocation during naphthalene metabolization.

CONCLUSIONS

Especially for microbial proteomics and the versatile metabolic potentials involved, alternative labeling strategies independent of cultivation conditions are needed. In this study, the suitability of 34S-sulfate as a labeling agent was successfully...
demonstrated. as the quantification results (i) fit the expected and reasonable physiological reactions and (ii) are in overall good agreement with the results from the supportive LFQ study. Our results indicate a very strong effect on the P. fluorescens proteome in response to naphthalene (see Fig. 6) and a clear oxidative-stress-like response with aldehyde dehydrogenase as a major NADPH provider and a specific set of proteases and chaperones to cope with protein misfolding and aggregation. Overall, the observed mechanisms seem to be very similar to the oxidative stress response reported for the Gram-negative bacteria F. nucleatum (39) or P. gingivalis (40). We further provide new computational tools for spectra extraction and disintegration of isotopic overlaps due to $^{34}$S incorporation. Further developments promise various additional applications in the future.

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