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# Ariadne's Thread in the Analytical Labyrinth of Membrane Proteins: Integration of Targeted and Shotgun Proteomics for Global Absolute Quantification of Membrane Proteins

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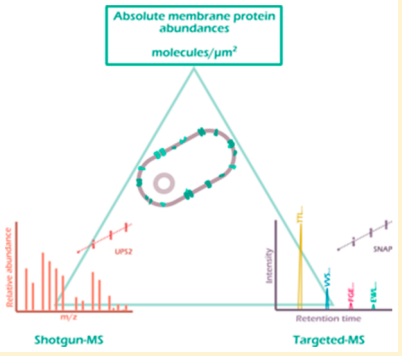
Supporting Information

**ABSTRACT:** The field of systems biology has been rapidly developing in the past decade. However, the data produced by "omics" approaches is lagging behind the requirements of this field, especially when it comes to absolute abundances of membrane proteins. In the present study, a novel approach for large-scale absolute quantification of this challenging subset of proteins has been established and evaluated using osmotic stress management in the Gram-positive model bacterium *Bacillus subtilis* as proof-of-principle precedent. Selected membrane proteins were labeled using a SNAP-tag, which allowed us to visually inspect the enrichment of the membrane fraction by immunoassays. Absolute membrane protein concentrations were determined via shotgun proteomics by spiking crude membrane extracts of chromosomally SNAP-tagged and wild-type *B. subtilis* strains with protein standards of known concentration. Shotgun data was subsequently calibrated by targeted mass spectrometry using SNAP as an anchor protein, and an enrichment factor was calculated in order to obtain membrane protein copy numbers per square micrometer. The presented approach enabled the accurate determination of physiological changes resulting from imposed hyperosmotic stress, thereby offering a clear visualization of alterations in membrane protein arrangements and shedding light on putative membrane complexes. This straightforward and cost-effective methodology for quantitative proteome studies can be implemented by any research group with mass spectrometry expertise. Importantly, it can be applied to the full spectrum of physiologically relevant conditions, ranging from environmental stresses to the biotechnological production of small molecules and proteins, a field heavily relying on *B. subtilis* secretion capabilities.




The past century was a successful period for molecular biosciences, as a great amount of data elucidating the function of individual molecules was produced. Despite its achievements, the results of this period came to corroborate the already present idea that, rarely, a biological function can be traced to a single molecule. Oppositely, most biological features are a result of intricate relationships between the cell's numerous components—genes, RNA molecules, proteins, and metabolites. Deducing and modeling this complexity is the focus of systems biology, aiming at a quantitative understanding of cellular systems.[1] These modeling endeavors are highly dependent on quantitative data, including those on protein abundances, as proteins represent the main carriers of biological activity and, hence, can provide answers regarding a high range of cellular processes. As systems biology approaches depend on absolute proteomic data rather than on relative comparisons of protein abundances, providing appropriate data represents a challenge for the scientific community.

Mass spectrometry (MS)-based proteomics has fundamentally reformed the way in which biological systems are questioned due to its capability to measure thousands of proteins in parallel.[2] Whereas a decade ago, most proteomic experiments predominantly provided a qualitative view of a biological system by enumerating its protein constituents, quantitative measurements are now inherent of practically every proteomic assay.[3] Thus, in the past few years there has been a rapid increase in the amount of relative and absolute protein data produced,[4−8] contributing to a great advance in

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the field of systems biology. Nevertheless, there are still many poorly understood traits. In particular, when it comes to absolute abundances of the membrane proteome, few if any data are available. This is mainly due to the characteristics of this specific subset of proteins, namely, their low abundance and their highly hydrophobic nature. However, due to the commitment of this specific protein class in crucial biological functions, there is a great need for a general method for absolute membrane protein quantification.

The here-described method addresses the issues inherent to absolute membrane protein quantification, by providing several control points throughout the workflow. To achieve this, two membrane proteins with different numbers of transmembrane domains (TMD)—4 and 13—were provided with the so-called SNAP-tag derived from the human alkylguanine-DNA alkyltransferase,[9] enabling the visualization of the hydrophobic fraction enrichment. The tag was chosen due to the availability of a wide range of possible substrates, thus enabling its adaption to the envisioned scientific question. In addition, absolute membrane protein concentrations were determined by integrating targeted mass spectrometric analysis of a SNAP-purified protein with quantification derived from calibrated shotgun proteomics data, where UPS2 human protein standards[10] were used to spike each sample. Fundamental to the method is that it relies on the application of a correction and an enrichment factor, which for the first time permit the calculation of absolute membrane protein abundances in a living organism.

## MATERIALS AND METHODS

**Bacterial Growth and Sample Preparation.** Strains and cloning strategies are detailed in the Supporting Information (paragraph "strain construction", Tables S-1 and S-2). For all proteomics analysis, the bacteria were grown in Belitsky minimal medium.[11] Exponentially growing cells (optical density at 500 nm [$OD_{500}$] of 0.4) were challenged with 6% (w/v) NaCl, and samples were taken 60 min after the onset of stress. Control cells, to which no NaCl was added, were collected at the same time point. Cells were harvested by centrifugation (10 000*g* for 15 min at 4 °C), and cell pellets were washed twice with TE buffer (20 mM Tris, 10 mM EDTA, pH 7.5). Cells were mechanically disrupted using the FastPrep24 instrument (MPBiomedicals), as it has proven to be the most efficient method for *Bacillus subtilis* cell disruption.[5] Cell debris was removed by centrifugation (20 000*g* for 10 min at 4 °C), and the protein concentration of the whole cell extract was determined by ninhydrin assay.[12] An aliquot with a protein content of 2.5 mg was used as starting material for membrane preparation. This lysate adjusted up to 1.5 mL of Tris EDTA buffer (10 mM EDTA, 20 mM Tris−HCl, pH 7.5) and subjected to ultracentrifugation (100 000*g* at 4 °C). The supernatant was discarded, and the pellet was detached from the bottom by adding 0.75 mL of high-salt buffer (10 mM EDTA, 1 M NaCl, 20 mM Tris−HCl, pH 7.5) and incubating in an ultrasonic bath for 5 min at room temperature. This was followed by pipetting the suspension up and down until the pellet was homogenized. The pipet was then rinsed with 0.75 mL of high-salt buffer, and the solution was incubated in a rotator at 8 rpm and 4 °C for 30 min, followed by ultracentrifugation under the same conditions as above. Pellet resuspension and ultracentrifugation were then performed with alkaline carbonate buffer (10 mM EDTA, 100 mM $Na_2CO_3$, 100 mM NaCl, pH 11), and in a final step with tetraethylammonium bromide (TEAB; 50 mM). The pellet containing the final crude membrane extract was resuspended in 50 μL of 1× SDS (sodium dodecyl sulfate) solubilization buffer (5% SDS, 50 mM TEAB, pH 7.55). The obtained pellet was designated as crude membrane extract, and 10 μg of material was used for protein digestion using the S-Trap protocol according to the manufacturer (ProtiFi). For shotgun-based absolute quantification, UPS2 proteins (Sigma-Aldrich-Merck) were added in a 1:4 ratio (2.5 μg). For liquid chromatography/mass spectrometry (LC/MS) analysis, 4 μg of peptide mixture per biological replicate was desalted using C18 Zip Tips (Merck Millipore). Peptide concentration was determined using the Pierce quantitative colorimetric peptide assay (Thermo Fisher Scientific). Preparation of whole cell and membrane extracts for targeted proteomics followed the same digestion protocol as described above, except for the addition of UPS2 standards. For each condition six biological replicates were processed belonging to the three different strains.

**LC/MS Data Analysis of Shotgun MS and Global Absolute Quantification of Membrane Proteins.** For data processing and protein identification, raw data were imported into MaxQuant (1.6.3.3)[13] incorporated with an Andromeda search engine,[14] and processed via the iBAQ algorithm.[10] Database searches were carried out against a reversed *B. subtilis* 168 database[15] with manually added SNAP and UPS2 sequences and with common contaminants added by MaxQuant. The database search was performed with the following parameters: peptide tolerance, 4.5 ppm; min fragment ions matches per peptide, 1; match between runs was enabled with default settings; primary digest reagent, trypsin; missed cleavages, 2; fixed modification, carbamidomethyl C (+57.0215); variable modifications, oxidation M (+15.9949), acetylation N, K (+42.0106). Results were filtered for a 1% false discovery rate (FDR) on spectrum, peptide, and protein levels. All identification and quantitation data are summarized in the Supporting Information (Table S-3), and the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[16] partner repository with the data set identifier PXD014272. Only proteins quantified in four out of six biological replicates were considered for further analysis.

**LC/MS Data Analysis of Targeted MS and Absolute Quantification of Native SNAP.** Raw files were processed using Skyline 4.2 (MacCoss Lab software[17]). On the basis of the added amount of purified SNAP protein, the absolute amount of native SNAP protein in both measured fractions was calculated. Absolute protein abundances derived from selected reaction monitoring (SRM) were compared to shotgun MS absolute protein abundances, and a correction factor was obtained by calculating a ratio between the targeted and the shotgun average concentration of native SNAP. In addition, an enrichment factor was attained by calculating the ratio between the median value of native SNAP in the membrane and whole cell extract fraction. This value allowed the subsequent calculation of protein copy numbers per total surface area (molecules per square micrometer), as it accurately provides the percentage of enrichment of the hydrophobic fraction and, thus, allows us to calculate back to the natural form of the membrane protein in the cell prior to enrichment. A final transition list for the SNAP protein is provided in the Supporting Information (Table S-4).

**Further Experimental Details.** Experimental details on determination of the bacterial cell size, Western blotting,



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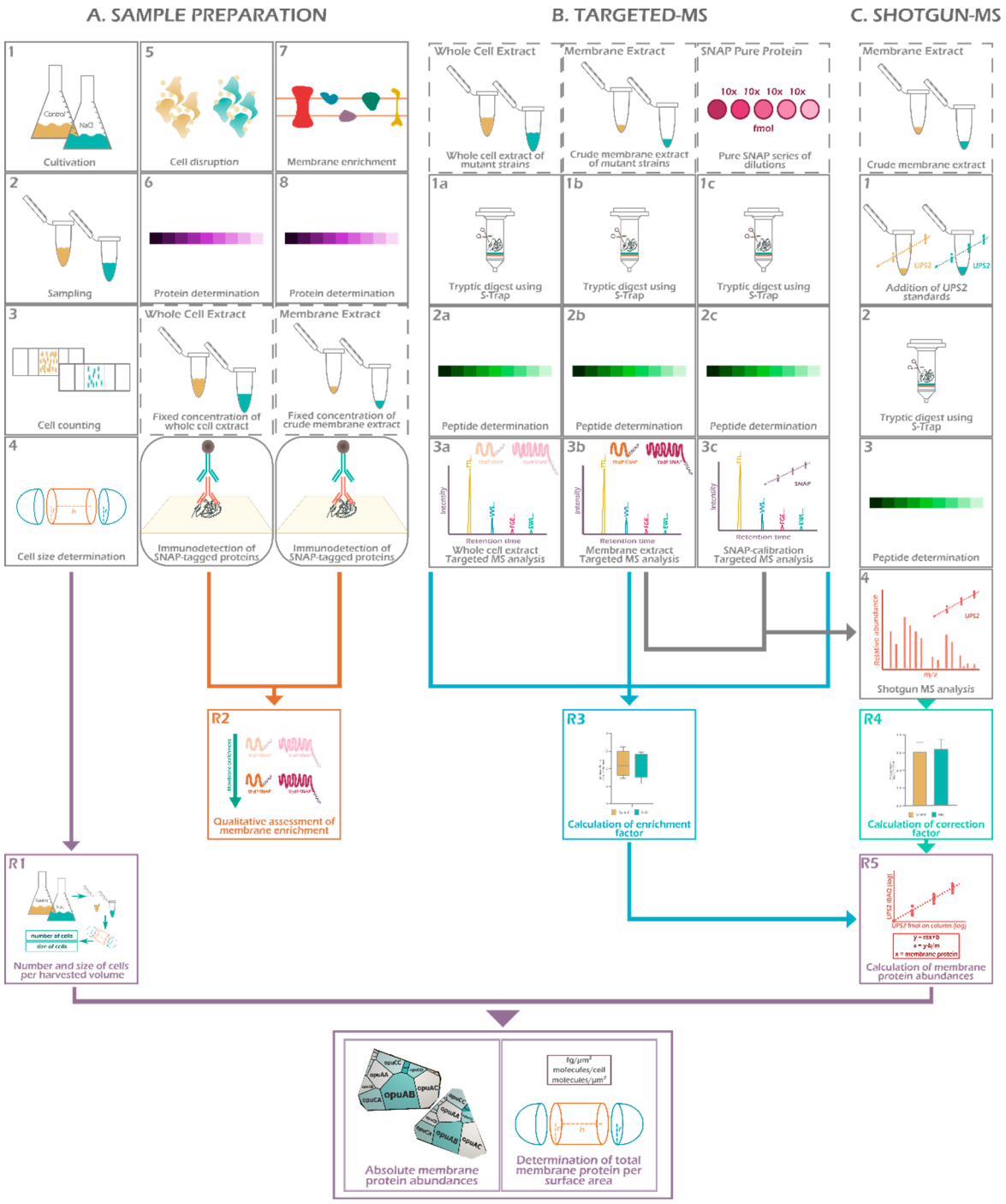


**Figure 1.** Workflow for absolute membrane protein quantification through calibration of shotgun MS by targeted mass spectrometry. (A) Steps involved in sample preparation. Filled lines refer to all experimental procedures, and irregular lines illustrate the resultant whole cell and membrane extracts derived from sample preparation. Rounded squares represent immunoassays performed during the workflow needed to visually confirm membrane enrichment, but not being part of the main sample preparation process. (B) Steps involved in targeted MS for each sample. Irregular lines illustrate the samples obtained from step A needed to conduct the targeted approach. Filled lines show the experimental procedure enumerating each consecutive step of the method. (C) Steps involved in shotgun MS analysis. Irregular lines correspond to samples used for the shotgun MS experiment and obtained from step A. Filled lines display the experimental procedure. "R" and the respective color make reference to the results ensuing the respective panel.

shotgun MS, and targeted MS analysis are provided as Supporting Information.

## RESULTS

Here we report, for the first time, a method exclusively developed for absolute membrane protein quantification in a living organism. This was achieved by optimizing previously established methods for absolute protein quantification and adapting them to the specific requirements imposed by the unique characteristics of membrane proteins. Shotgun proteomics was combined with the usage of spiked-in internal standards (UPS2) prior to membrane fraction digestion,



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allowing for the calculation of absolute membrane protein abundances. Nonetheless, in order to calculate the number of protein molecules per square micrometer of membrane area, it was essential to calculate an enrichment factor, as the membrane-enriched protein fraction does not reflect the cell's membrane proteomome in its native state. In addition, a correction factor was also calculated, as UPS2 standards do not necessarily mimic the physicochemical properties of membrane proteins. To do so, two different membrane proteins were chromosomally tagged using the SNAP-tag and the abundances of these proteins were measured before and after membrane enrichment by measuring SNAP-tag protein absolute abundances by targeted proteomics. The experimental pipeline is graphically represented in Figure 1.

To demonstrate the accuracy of our method we chose *B. subtilis* 168, the model for Gram-positive bacteria, exposed to osmotic shock as a proof of principle. In particular, protein concentrations were determined 1 h after the onset of stress, thereby comparing control and stress conditions. Moreover, as this study was dedicated to the study of the membrane fraction, the absolute numbers presented here were focused on the membrane protein data set of this organism.

**Optimization of Shotgun-Based Absolute Quantification and Sample Preparation for Global Quantification of Membrane Proteins.** Label-free MS approaches have shown to be best suitable for large-scale absolute protein quantification.[18] Hence, in order to develop the described method, a widely accepted method for global absolute quantification was tested—iBAQ[10]—by analyzing a total protein extract of *B. subtilis*. This method uses the sum of all peptide peak intensities of a sample divided by the number of theoretically observable tryptic peptides as indicator of protein abundances. This approach showed a linearity in quantification of 4 orders of magnitude with the UPS2 standards, and a very good correlation ($r^2$ = 0.9503) (Figure S-1), thus being the method used in this study. Furthermore, in order to ensure efficient digestion of the crude membrane extract, several digestion protocols were tested—in-solution,[5] filter-aided sample preparation (FASP),[19] and suspension trapping (S-Trap).[20] Also, accuracy and sensitivity of quantification was tested by spiking in UPS2 standards in each sample. The method that provided the highest number of membrane protein identifications was S-Trap, with a total of 516 membrane proteins, followed by FASP and in-solution digest, which identified 495 and 473 membrane proteins, respectively (Table S-5). As for sensitivity and accuracy of membrane protein quantification, all methods enabled the quantification of 4 orders of magnitude of UPS2 standards, with FASP showing the highest correlation, followed by S-Trap and in-solution digest (Figure 2A). Nevertheless, it has been reported that the FASP approach suffers from batch-to-batch variation,[20] as this method relies in the use of a membrane filter hindering its application in high-throughput proteomic studies, and thereby suggesting that it might not be the most adequate method for the purpose of this study. Furthermore, we compared the overlap between the quantified membrane proteins for all the tested methods. For this purpose, values were only considered valid if present in all three biological replicates. The results show a considerable overlap between the three approaches, with S-Trap providing the highest number of quantified membrane proteins (Figure 2B). A recently published study has also compared these three digestion methods and has shown that the most efficient digestion

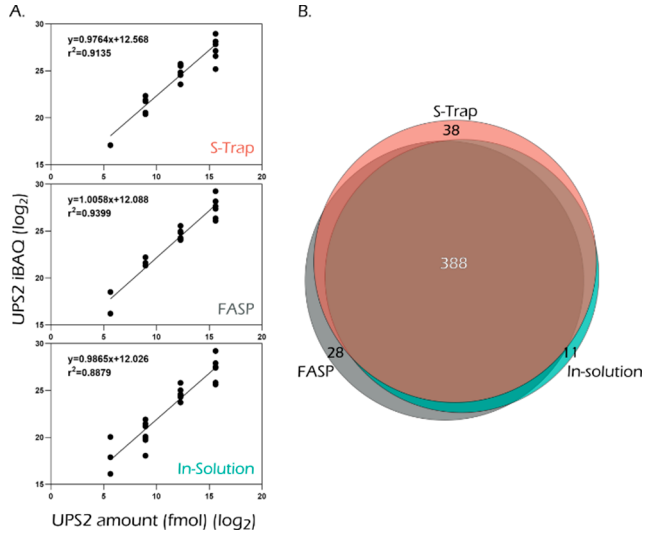


**Figure 2.** Comparison of three digestion methods—S-Trap, FASP, and in-solution. (A) Linear regression of UPS2 standards quantified in the three tested digestion methods with respective correlation. (B) Overlap of the quantified membrane proteins in all three biological replicates between the digestion methods.

protocol was S-Trap, as it provided the best overall performance, with the highest number of protein identifications, reproducibility of quantification, and sensitivity,[21] which is well in accordance with our data (Figure 2 and Table S-5). Also, since S-Trap digestion allows for a slightly higher concentration of SDS (5%) in comparison to FASP and in-solution digest, it was the chosen method for membrane protein digestion.

**Accurate Absolute Membrane Quantification Workflow.** To adapt an absolute quantification approach to the specificities of membrane proteins, we applied the SNAP-technology. The SNAP-tag served two functions: (1) qualitative assessment of the enrichment of the membrane fraction, and (2) calibration of the shotgun proteomics absolute data (Figure 1, results R3 and R4). We chromosomally tagged two *B. subtilis* membrane proteins with different numbers of predicted TMD[22]—YodF (unknown function and with 13 TMD) and YhdP (responsible for magnesium export and with 4 TMD)—in order to have a quantification method valid for different classes of membrane proteins. The nontagged parental version of *B. subtilis* was also used for quantification in order to verify that absolute protein abundances were not affected by the insertion of the tag. It is of course unfeasible to tag all membrane proteins of this organism, but we believe that tagging differentially abundant proteins, which have different molecular weights and varying numbers of TMD, provides already a fair representation for quantification and proof-of-principle purposes. To ensure that the tag did not have an effect on bacterial growth, we compared the growth curves of all the three strains, both in control and osmotic shock conditions, and no difference was observed (Figure S-2).

The qualitative assessment of the enrichment of membrane proteins was achieved by loading the whole protein and membrane extract for both conditions (control and NaCl) on a SDS gel, and then detecting the tagged proteins by immunoassays (Figure 1, result R2). In order to test the limit of detection of the SNAP protein and also the specificity



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of the anti-SNAP antibody we conducted immunoassays and verified that the anti-SNAP antibody is highly specific toward the SNAP protein (Figure S-3A). We also observed that the limit of detection of the SNAP protein is in the range of 25 ng (Figure S-3B), allowing us to detect the tagged proteins in very low concentrations and in a highly specific manner. The immunoassays for YodF- and YhdP-tagged proteins in the two different conditions were performed in triplicates and showed a consistent enrichment of the membrane fraction, independent of the number of transmembrane domains (Figure S-4, parts A and B). Second, the pure SNAP protein served as anchor protein for targeted MS analysis and further calibration of the absolute protein abundances obtained from the conversion of iBAQ intensities to molar amounts for all identified membrane proteins (Figure 1, results R3, R4, and R5). This was achieved by measuring a calibration curve of the purified SNAP protein ranging 5 orders of magnitude (0.001−10 pmol on column) by SRM (Figure 1B, step 3c, and Figure S-5). The calibration was based on six transitions of three peptides weighted according to their area-to-background (A/B) ratios before being averaged over the peptide AUC (area under the curve) intensities to result in the calculated absolute abundance of the SNAP protein. Then, the log-transformed weighted averages of AUC intensities were plotted against known log-transformed absolute amounts of the SNAP-purified protein. The SNAP calibration curve shows the sensitivity and wide dynamic range of the SRM approach as this method enabled the accurate quantification of three peptides over 5 orders of magnitude and with an $r^2$ of 0.9985 (Figure S-5). This calibration enabled us to calculate absolute amounts of native SNAP in the respective strains before (whole-cell extract) and after (crude membrane extract) enrichment (Figure 1, result R3).

**Calibration of Shotgun MS Results Using Targeted Proteomics.** The pure SNAP protein served as anchor to calculate absolute amounts of its native form in the chromosomally tagged strains, in order to allow the calculation of the concentration for the two different tagged membrane proteins by targeted MS (Figure 1, result R3). The slope and intercept from this calibration curve were used to convert SRM-based weighted AUC intensities of the chromosomally SNAP-tagged strains to absolute molar amounts. Four biological replicates of digested whole cell and membrane extract were measured for each condition, control and 6% NaCl (w/v), and absolute amounts of the SNAP-tagged protein were calculated.

We calculated the ratio between native SNAP absolute molar amounts of membrane and total cell extract in order to determine the enrichment factor between whole cell extract and enriched membrane protein sample (Figure 1, result R3). This resulted in values of 4.40 and 5.02 for control and NaCl, respectively (Figure S-6A). This is the quantitative corroboration of what is already visible in the immunoassays (Figure S-4)—an efficient enrichment of the membrane fraction regardless of the number of TMD. In addition, the SRM results show that there is a slightly higher enrichment in the osmotically stressed cells. Remarkably, this same tendency is shown by our ninhydrin-based protein determination assay, in which the control replicates have a marginally lower concentration than the osmotically challenged cells (Table S-6).

The SRM approach was used to calibrate the shotgun-derived absolute data by applying a correction and an enrichment factor, both being essential to develop an accurate calculation for absolute membrane protein quantification due to the intrinsic hindrances involved in the handling of this subset of proteins (Figure 1, result R5, and Figure S-6). Determination of a correction factor was achieved by calculating a ratio between the absolute molar amounts of the native SNAP protein obtained in the SRM approach and its shotgun counterpart (Figure 1, result R4). We calculated a median ratio of 0.622 and 0.654 for control and NaCl, respectively (Figure S-6B). This shows that, even though the UPS2-based absolute quantification is very accurate, it still provides a slight overestimation of total protein abundances. Moreover, this overestimation does not appear to be condition-dependent, as the calculated correction factor is similar for both tested conditions.

Both the correction and enrichment factor were then used to calibrate the data obtained by the shotgun approach.

**Determination of Cellular Protein Concentrations.** Cell counting was performed at the moment of harvesting; thus, every sample was analyzed taking into account the number of cells present in a given volume of medium. As incomplete cell lysis might represent a possible source of error, the disruption method as developed by Maaß et al. was employed, as it has proven to provide disruption efficiencies better than 99% for *B. subtilis*.[5] With this sample disruption efficiency and knowing the number of cells per volume of culture, the determination of protein copy numbers per surface area was possible. This value was calculated after accurate determination of the average size of *B. subtilis* cells in the two tested physiological conditions using light microscopy (Figure 1, result R1). Absolute protein amounts per microgram of crude membrane extract, protein concentrations, copy numbers per surface area, and molecules per cell for all membrane proteins quantified by shotgun MS are presented in the Supporting Information (Table S-3). A table showing the average sizes of all measured *B. subtilis* cells per condition is also available in the Supporting Information (Table S-7).

Absolute membrane abundances were calculated by plotting the log-transformed iBAQ intensities against known log-transformed absolute molar amounts of the spiked-in UPS2 standards.[10] The resulting linear regression was used to fit iBAQ intensities to absolute standard protein amounts. The slope and intercept from this calibration curve were then used to convert iBAQ intensities of all identified *B. subtilis* proteins to molar amounts. This enabled the quantification of 4 orders of magnitude for the UPS2 standards for both control and stressed cells, with an $r^2$ of 0.9753 and 0.9624, respectively (Figure S-7). After determination of absolute molar amounts of the quantified proteins these values were calibrated by applying both the correction and enrichment factor derived from the SRM approach (Figure 1, result R5).

**Biological Significance of Determined Membrane Protein Concentrations.** Our study provides, for the first time, a method exclusively developed for absolute membrane protein quantification in a living organism. Consequently, there are currently no absolute membrane quantification studies available for any bacteria, and thus, comparison with published data is impossible. To corroborate the accuracy of this newly developed approach, the determined absolute protein concentrations were therefore compared to other types of data from previously published physiological studies. In this study we determined that the ATP synthases subunits—AtpF and AtpE—are the most abundant proteins in *B. subtilis* with about 160 molecules/$\mu m^2$ each during exponential phase for



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both control and stress conditions. Consistent with this observation, a previous study investigating the transcriptional architecture of *B. subtilis* reported that genes encoding enzymes involved in ATP synthesis are among the most highly expressed genes and also among the least tightly regulated ones.[23] This is well in accordance with the present data, as AtpF and AtpE have a similar abundance under both tested conditions (Table S-3). The present quantitative approach also uncovered a wide dynamic range for low-abundant membrane proteins, where the values ranged between 100 and 0.05 copies/$\mu m^2$.

Details on the assignment of membrane protein copy numbers per cell surface to a specific cellular function are presented in the Supporting Information (Figure S-8A). By far, the most abundant group of membrane proteins are transporters, with ∼19% of the quantified protein molecules being assigned to this functional category. This reflects the versatility of transport systems in this organism and is consistent with the qualitative results from a previous study targeting the membrane proteome of *B. subtilis*.[24] Furthermore, our data shows that ∼12% of the quantified membrane proteins are involved in stress management. Interestingly, our results show that, when it comes to coping with hyperosmotic shock, the cells dedicate 4% more of their "cellular budget" to coping with the consequences of the hyperosmotic stress as compared to the control cells (Figure S-8B).

Additional support for the reliability of our quantification method can be derived from the known physiological responses of *B. subtilis* to imposed salt stress. According to previous investigations, the initial response of this organism to acute osmotic stress relies on the uptake of large amounts of potassium ions, followed by a phase of adaptation in which compatible solutes such as proline and betaine are accumulated via synthesis and uptake.[25] Accordingly, we observed that *B. subtilis* dramatically increased the copies of GltA, the large subunit of the glutamate synthase, upon salt stress (Figure 3, Figure S-9 and Table S-3). This could be explained by the imposed deprivation of glutamate, a precursor for proline synthesis, from the Belitsky minimal growth medium used in our present study. As a consequence, *B. subtilis* try to synthesize new molecules of glutamate to be able to produce the compatible solute proline. Also, the data shows a general increase in the copy numbers of proteins belonging to the Opu family, with a clear predisposition for the OpuA operon (opuAA−opuAB−opuAC) which mediates the uptake of glycine betaine. OpuE, necessary for the uptake of proline, also shows a significant increase. However, it is present in much lower copy numbers than the other proteins of the Opu family. This might relate to the fact that proline is the only compatible solute used by *B. subtilis* that can also be exploited as a nutrient, limiting the effectiveness of exogenously provided proline as an osmostress protectant.[26] As a consequence, this organism might give preference to more efficient uptake systems for compatible solutes, like OpuA, present in higher abundances (Figure 3 and Table S-3). A table with all membrane protein abundances significantly changed during osmostress is available in the Supporting Information (Table S-8).

Lastly, our quantitative membrane proteome data can be applied to assess the stoichiometry of membrane protein complexes (Table 1). For example, our method reports a ratio of 1:1 as opposed to 2:1 for the components of the stator of the flagellar motor MotA:MotB in *Escherichia coli*.[27] This

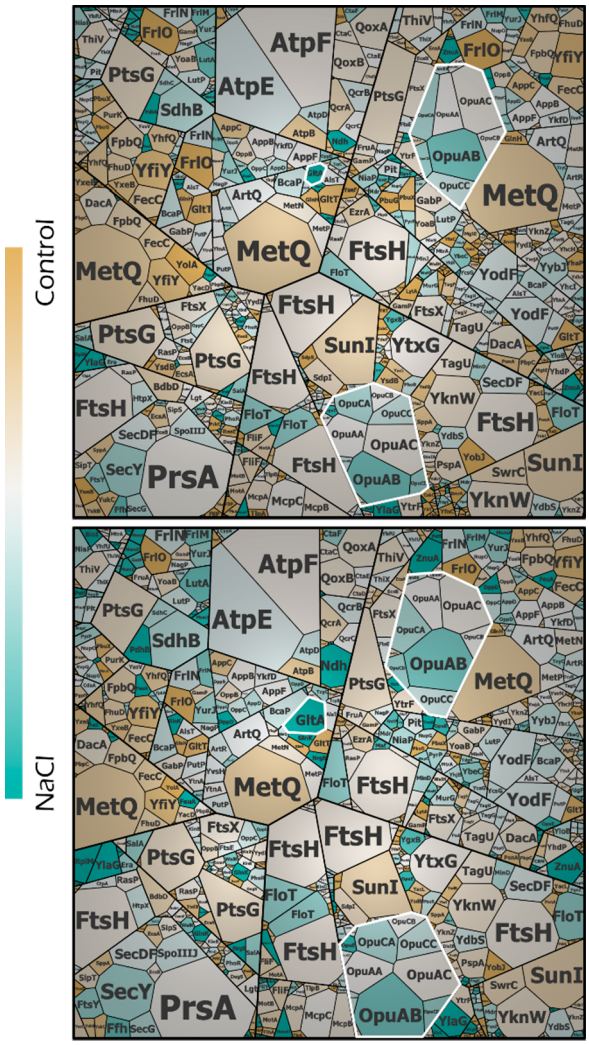


**Figure 3.** Voronoi treemaps illustrating copy numbers per square micrometer of membrane proteins in stress and control conditions. Proteins quantified via shotgun MS are displayed as single cells, which are functionally clustered according to the SubtiWiki gene orthology (ref 36). A protein appearing more than once is included in more than one functional category. GltA and Opu family of transporters are highlighted for ease of visualization. Cell size corresponds to protein abundance, and color code indicates abundance in each of the measured conditions: brown, proteins more abundant in control conditions; blue, protein more abundant in stress conditions; white, no difference in protein abundance.

suggests a different architecture of the flagellar motor in *B. subtilis*. We also compared the stoichiometry of the Sec system for protein translocation across the membrane with previously published data. This showed that accessory components SecDF and SpoIIIJ (MisCA) are present in about the same amounts as the main translocation channel component SecY, which is in agreement with previously published data for *E. coli*.[28] On the other hand, the SecE channel component was not detected and SecG was detected in 4-fold lower amounts than SecY. The latter could be due to the fact that SecG of Gram-positive bacteria may be poorly retained in the channel and released into the medium.[29] Lastly, a recently performed study suggested a putative complex between the signal peptide peptidase SppA and the stress protein YteJ, hinting that SppA is 2 times more abundant than YteJ (G. Henriques, O.



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Table 1. Stoichiometry Information for Selected Proteins[a]

| | *B. subtilis* | literature | organism |
|---|---|---|---|
| MotA:MotB | 1.0 ± 0.3:1.0 ± 0.2 | 2:1 | *E. coli* (ref 27) |
| SecDF:SecG:SecY:SpoIIIJ:YrbF | 1.0 ± 0.1:0.4 ± 0.1:1.7 ± 0.5:1.0 ± 0.2:3.4 ± 1.8 | 1:1:1:1:1:1 | *E. coli* (ref 28) |
| SppA:YteJ | 3.7 ± 0.2:1.0 ± 0.1 | 2:1 | *B. subtilis* (P.C.) |

[a]Stoichiometry composition of known protein complexes was determined using the absolute quantification workflow herewith described and compared to previous observations (column "literature"). The standard deviation between replicates is also presented in the table. Literature values were extracted from the indicated references as well as the organism in which these studies were performed. P.C. stands for personal communication.

Delumeau, M. Jules, personal communication). This result is well in accordance with our findings.

## DISCUSSION

Here we report the first methodology for absolute quantification of membrane proteins as exemplified with the model Gram-positive bacterium *B. subtilis*.

One of the most crucial steps in membrane preparation is the enrichment of this fraction. This was accomplished by washing isolated membranes with different buffers that favor the precipitation of hydrophobic proteins and, at the same time, allow the consequent depletion of their soluble counterparts. Importantly, our approach tackled this bottleneck for absolute membrane protein quantification by providing two control points—immunoassay and targeted proteomics—which ensure the correct determination of the membrane fraction enrichment, independently of the number of TMD contained in proteins belonging to this subcellular fraction. In addition, samples were digested using the S-Trap technology,[20] which combines the advantage of efficient SDS-based protein extraction with rapid detergent removal, thereby enabling an efficient solubilization of membrane proteins and making them more accessible for proteolytic digestion. This innovative implementation led to identification of 496 membrane proteins of *B. subtilis*, of which 231 contain four or more TMD. Of the remaining 265 membrane proteins, 105 have no TMD according to the HMMTOP2.0 prediction tool,[22] suggesting that they are likely membrane-associated proteins. The number of membrane protein identifications is higher than the one reported by previous studies targeting the membrane of *B. subtilis*,[24,30,31] which is most likely due to the employed digestion method and the usage of faster and more sensitive mass spectrometers. Nonetheless, there is still room for improvement, as the present study covers ∼40% of the predicted membrane proteome of this organism. In this respect it is noteworthy that a recently published study employed a coacervate-based differential phase method to enrich hydrophobic proteins of yeast, resulting in 13% more identifications of integral membrane proteins and 25% more identifications of low-abundant proteins.[32] Our present methodology for absolute membrane protein quantification could probably be combined with this recently developed membrane enrichment method, but the correction and enrichment factors developed in this study would still be essential to accurately determine membrane protein concentrations.

The protocol for absolute membrane protein quantification developed in this study makes use of two chromosomally SNAP-tagged membrane proteins with 4 and 13 TMD, in order to cover a broad spectrum of membrane proteins with different physicochemical properties. This allowed for the extrapolation of their behavior to the rest of the proteins belonging to this subcellular fraction. One might argue that, in order to achieve a more comprehensive data set, more representatives of this group of proteins should be studied in similar detail. However, the present results show that the enrichment factor is similar for membrane proteins with different physicochemical properties (4 and 13 TMD), indicating that additional SNAP-tagged membrane protein representatives would not contribute further insights.

As membrane proteins are generally present in lower abundances in comparison to soluble proteins, one could also argue that data-independent acquisition (DIA) would comprise a suitable technique to absolutely quantify membrane proteins, especially since all peptides within a defined mass-to-charge ratio are subject to fragmentation, in contrast to data-dependent acquisition (DDA) where the mass spectrometer is inherently biased to pick for fragmentation those peptides with the strongest signal.[33] However, a study comparing DIA and DDA reported that, in low-complexity UPS2 samples, both methods identified similar numbers of peptide ions and proteins, with DIA identifying only more peptide ions than DDA only for higher-abundant proteins.[34] Thus, we believe that DDA is a sufficiently powerful method to meet the requirements of absolute membrane protein quantification. Regardless, the absolute quantification of membrane proteins will certainly benefit from the endeavors that are currently being dedicated to different data acquisition methods and instrumentation in the vast world of mass spectrometry.

The workflow here described comprises a highly comprehensive and accurate method to determine membrane protein concentrations in an absolute manner, a methodology not available until now. The resulting information is essential for systems biology investigations, since this field relies on detailed knowledge of the concentrations of expressed proteins as a function of the cellular state in order to build mathematical models that simulate biological processes.

Even though this newly developed method does not cover the entirety of the membrane proteome of *B. subtilis*, it is capable of accurately detecting the physiological changes resultant of an imposed stress, offering a clear visualization of alterations in protein arrangements. The straightforwardness of our method allows it to be easily applied to any type of physiological condition. This will enable researchers to address different types of research questions, for instance, in the biotechnological sector, which is in need of detailed quantitative information on cellular responses at the level of the membrane to fully understand the consequences of secretion stress.[35]

Lastly, it should be noticed that membrane protein quantification is probably more prone to error than the quantification of soluble proteins, due to the physicochemical properties of membrane proteins. However, the precision of the introduced approach (comparison Table 1) is in full accordance with recently reported MS-based approaches


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targeting soluble proteins[5−7] (approximately 2-fold error among 3 orders of magnitude), which corroborates the accuracy of the here presented protocol.

## CONCLUSION

Recent developments in the mass spectrometry field have allowed the successful determination of absolute abundances of soluble proteins.[4−8] However, the quantification of their hydrophobic counterparts in biological membranes has until now failed to succeed. The described workflow represents a straightforward approach for absolute membrane protein quantification. It tackles the crucial bottlenecks involved in the handling and preparation of this fascinating, but technically challenging, class of proteins. Our novel approach combines the accuracy and sensitivity of targeted MS with the resolving power and comprehensiveness of shotgun MS, thereby providing access to cellular membrane protein concentrations for a large subset of membrane proteins. We believe this approach will help to answer long-standing questions of the scientific community regarding membrane protein dynamics in response to physical, chemical, and physiological perturbations, that are both of fundamental scientific and biotechnological interest.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b02869.

Strain construction, determination of cell size, Western blotting, shotgun MS and targeted MS analysis, calibration curves, growth curves, immunoassay results, enrichment and correction factors obtained by SRM measurements, assignment of membrane protein copy number per cell surface to a specific cellular function, Voronoi treemaps, lists of strains used in this study, template DNA and respective primers used for strain construction, all membrane proteins quantified in this study, sequences of MS-quantified SNAP peptides and their optimized SRM acquisition parameters, number of identified proteins in tested digestion methods for each subcellular fraction, ninhydrin assay results, cell dimensions and cell surface area, and a list of membrane protein abundances significantly changed during osmostress (PDF)

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### Author Contributions

M.A.-V., J.B., A.O., S.M., and D.B. conceived and designed the experiments. M.A.-V. performed experiments and analyzed the data. A.Q.-G., K.A., and A.N. designed the primers and performed strain construction. M.B.-C. contributed reagents and cloning strategy. T.S. performed the sample measurement. S.M., A.O., A.N., M.R., J.M.v.D., and D.B. supervised the project. M.A.-V. wrote the manuscript, and S.M. and D.B. provided all necessary corrections. All authors have read and approved the manuscript.

### Notes

The authors declare no competing financial interest.

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