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Improving Proteome Coverage for Small Sample Amounts: An Advanced Method for Proteomics Approaches with Low Bacterial Cell Numbers

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Proteome analyses are often hampered by the low amount of available starting material like a low bacterial cell number obtained from in vivo settings. Here, the single pot solid-phase enhanced sample preparation (SP3) protocol is adapted and combined with effective cell disruption using detergents for the proteome analysis of bacteria available in limited numbers only. Using this optimized protocol, identification of peptides and proteins for different Gram-positive and Gram-negative species can be dramatically increased and, reliable quantification can also be ensured. This adapted method is compared to already established strain-specific sample processing protocols for *Staphylococcus aureus, Streptococcus suis,* and *Legionella pneumophila*. The highest species-specific increase in identifications is observed using the adapted method with *L. pneumophila* samples by increasing protein and peptide identifications up to 300% and 620%, respectively. This increase is accompanied by an improvement in reproducibility of protein quantification and data completeness between replicates. Thus, this protocol is of interest for performing comprehensive proteomics analyses of low bacterial cell numbers from different settings ranging from infection assays to environmental samples.

Comprehensive proteome analyses of host pathogen interactions in animal models, human specimen, or infection mimicking cell culture experiments are often hampered by the low number of available bacterial cells. Furthermore, bacterial components need to be analyzed in the presence of a huge excess of host material. This is particularly pronounced for proteins, which display a greatly varying dynamic range in vivo. To overcome this problem, different strategies for enrichment of bacteria from infection assays[1–6] as well as environmental samples[7–13] were developed in order to increase the number of detected bacterial proteins. However, not only enrichment of bacteria is of importance, but also optimized cell lysis[14] and sample preparation for LC–MS/MS analysis. In the last years, different sample preparation methods have emerged and been adapted.[15] However, each additional processing step, e.g., removal of interfering compounds, results in sample loss, particularly when starting with low cell numbers, severely compromising the number of proteins covered. The single pot solid-phase enhanced sample preparation protocol (SP3 protocol) uses magnetic beads for the sample preparation and was first introduced by Hughes et al.[16] This protocol displays increased efficiency, speed, throughput, and robustness to, e.g., solvents, pH, or sample complexity for sample processing in proteomics of eukaryotic cells.[17,18] Sielaff et al. published an slightly improved protocol in 2017 that enabled identification of about 3400 proteins and up to 30 000 peptides from as low as 1 µg protein from HeLa cells.[19] However, so far no studies are available that apply the SP3 protocol to bacteria. When only low numbers of bacteria can be recovered it is crucial to use a harsh but efficient cell disruption, without interfering with subsequent sample preparation. The SP3 protocol provides the option to easily remove compounds required for efficient cell disruption but incompatible with subsequent analysis by MS. In order to proof applicability to diverse bacteria, we combined the SP3 protocol introduced by Sielaff et al.[19] with optimized cell disruption protocols for the Gram-negative bacterium *Legionella pneumophila* Corby, the non-capsulated Gram-positive pathogen *Staphylococcus aureus*, and the capsulated Gram-positive veterinary pathogen *Streptococcus suis*. In all three cases, we observed increased peptide coverage and thus, enhanced protein identification as well as more reliable and reproducible protein quantification with the adapted SP3 protocol in comparison to the strain specific standard protein preparation and in-solution digestion procedures applied before.

S. aureus NCTC8325-4,[20] *L. pneumophila* Corby (kindly provided by Bernd Schmeck, University Medicine Gießen/Marburg,

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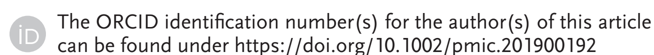
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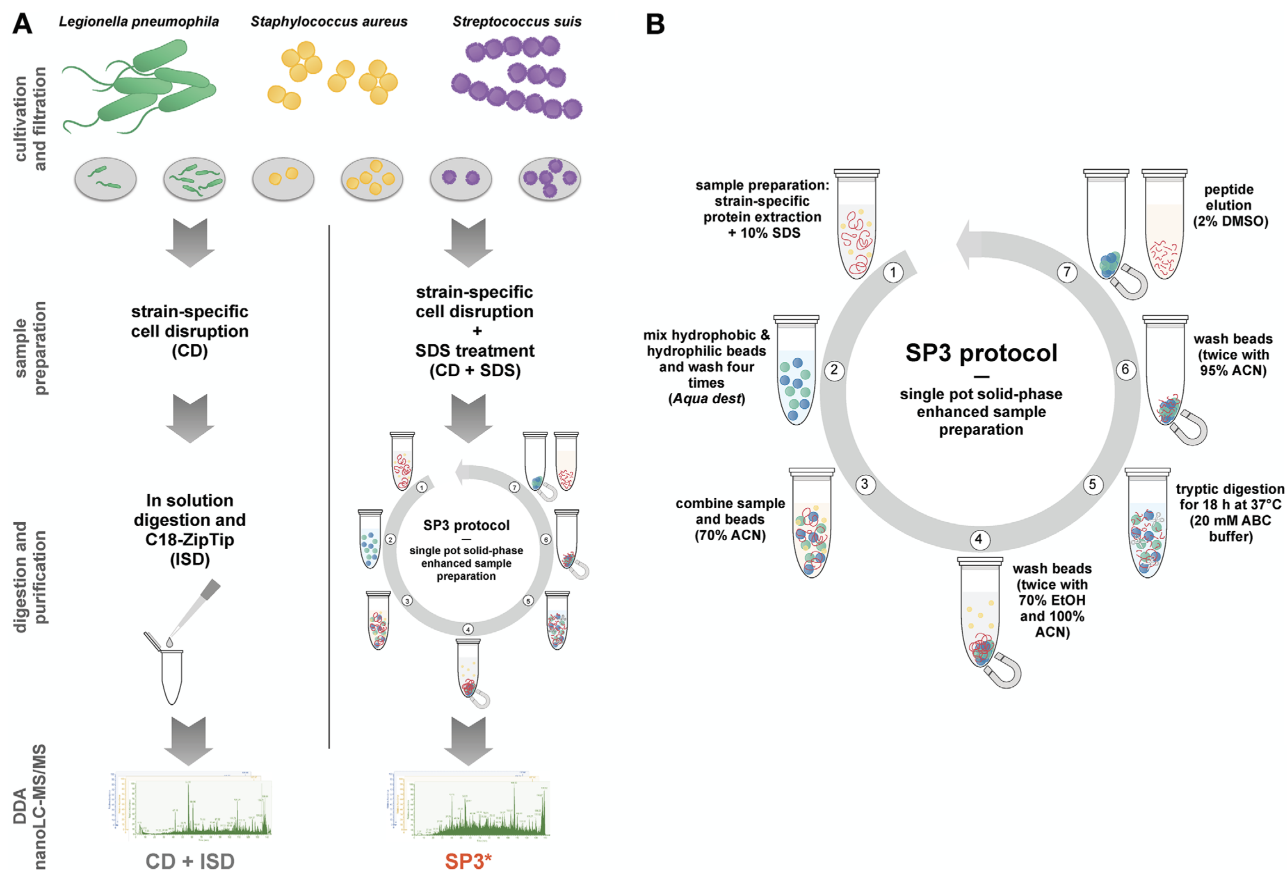

Figure 1. A) Comparative overview of workflows of the optimal strain specific disruption with in-solution digest (CD+ISD) and the optimized SP3 protocol (SP3*). Both protocols were tested with three different bacteria, Gram-negative *L. pneumophila*, Gram-positive *S. aureus*, and a capsuled Gram-positive *S. suis*. Each bacterium was cultivated in shake flasks until mid-exponential phase and 2×10^6 or 5×10^6 cells were collected on a filter. Cell disruption was performed in a species-specific manner and samples were in-solution digested with trypsin and purified with a C18 ZipTip (CD+ISD) or samples were disrupted with a combination of SDS treatment and species-specific disruption and purified via the SP3 protocol (SP3*). All samples were measured on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, MA, USA) in DDA mode. B) Schematic presentation of the different processing steps of the newly adapted SP3 protocol (SP3*). Starting with a mixture of detergent, salts, or enzymes from species-specific cell disruption and bacterial proteins (1), and prepared beads (2), samples were added to an appropriate volume of beads, depending on protein amount of samples (3). Sample-bead-mixes were washed several times with EtOH or ACN (4). Afterward a tryptic digestion in ABC-buffer at 37 °C was performed over night for 18 h (5). Additional washing steps with ACN were performed to remove of ABC-buffer and excessive trypsin (6). Finally, peptides were eluted by adding 2% DMSO (7) (B).

Germany), and the *S. suis* strain 10 (serotype 2) isolate (kindly provided by Hilde Smith, DLO-Lelystad, The Netherlands) were cultivated in the appropriate medium and conditions according to Table S1, Supporting Information and harvested in the exponential growth phase. In order to allow flow cytometry-based cell counting, a green fluorescent protein (GFP) expressing *L. pneumophila* Corby strain was used. For the analysis of *S. aureus* NCTC8325-4 and *S. suis* the bacterial cultures were stained using SYTO9 (Thermo Fisher Scientific, MA, USA) as described before.[21] After determining the bacterial cell numbers in each culture using a Guava easyCyte flow cytometer (Merck-Millipore, MA, USA), 2×10^6 or 5×10^6 bacteria, respectively, were collected on 0.22 µm filters (96-Well Durapore membrane filtration plate Multiscreen HTS, Merck, Germany) using a vacuum pump (≈200 mbar, KNF Laboport, NJ, USA). Filters were cut, transferred into reaction tubes, and stored at −80 °C until further use.

Subsequent proteomics experiments were performed in low-protein binding 1.7 mL reaction tubes in triplicates to compare standard strain-specific cell disruption and in-solution digestion (CD+ISD) with the adapted SP3 protocol (SP3*: cell disruption with additional SDS treatment and subsequent SP3 preparation; **Figure 1**).

For the CD+ISD preparation *L. pneumophila* cells on filters were dissolved in 20 µl aqueous buffer containing 8 mol L^{-1} urea and 2 mol L^{-1} thiourea (UT-buffer) and incubated two times for 30 min (23 °C, 1400 rpm on an Eppendorf ThermoMixer (Eppendorf, Germany)) with subsequent ultra-sonication for 3 min in an ultrasonic bath (Sonorex, Bandelin, Germany). The samples were centrifuged and the supernatant containing the proteins was transferred into a new reaction tube. In order to decrease the urea concentration for efficient trypsin digestion, 60 µL aqueous ammonium bicarbonate solution (20 mmol L^{-1}; ABC-buffer) were added. *S. aureus* proteins were extracted with lysostaphin and digested with trypsin as described before.[22] *S. suis* on filters were incubated for 30 min, at 37 °C, 1400 rpm (Eppendorf ThermoMixer, Germany) with 30 µL ABC-buffer (50 mmol L^{-1})

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containing 2.5 ng lysozyme to disrupt the cells. Subsequently, protein extraction was enhanced by 3 min treatment in an ultrasonic bath. After spinning down the sample, the supernatant containing the proteins was transferred into a new reaction tube. For digestion of protein extracts of 2×10^6 bacterial cells 40 ng trypsin (Promega, WI, USA) and for those of 5×10^6 bacterial cells 80 ng trypsin were added and the samples were incubated for 18 h at 37 °C. Digestion was stopped with a final concentration of 1% (v/v) HPLC-grade acetic acid. The resulting peptides were purified using C_{18} ZipTip columns (Mettler Toledo, Germany) and subsequently solvents were removed by lyophilization. The dried peptides were stored at −80 °C and reconstituted in buffer A containing 2% (v/v) acetonitrile (ACN; Thermo Fisher Scientific, MA, USA) and 0.1% (v/v) acetic acid (Carl Roth GmbH & Co. KG, Germany) in HPLC-grade water (J.T. Baker, Thermo Fisher Scientific) for LC–MS/MS measurement.

For the adapted SP3 protocol (SP3*), the following procedure was applied. Samples were disrupted as described above (CD) but filters were additionally rinsed with 30 µL 10% (v/v) SDS to improve cell disruption. SDS and CD suspension were combined. In parallel to the cell disruption, hydrophobic and hydrophilic magnetic beads [hydrophobic: Sera-Mag Speedbeads carboxylated-modified particles [(Thermo Fisher Scientific); hydrophilic: Speedbead magnetic carboxylated modified particles (GE Healthcare, United Kingdom)] were prepared freshly before usage as described by Hughes et al.[16] The protein suspension was mixed with an appropriate amount of beads (2 µL beads μg^{-1} protein) and ACN to reach a final ACN concentration of 70% (v/v). Samples were incubated at room temperature for 18 min at 1400 rpm on an Eppendorf ThermoMixer® (Eppendorf, Germany) to facilitate binding of proteins to the beads. After 2 min incubation on a magnetic separation rack for bead sedimentation, supernatants were removed. Next, beads were washed two times with 180 µL 100% (v/v) ethanol and then twice with 180 µL ACN, with intermittent bead sedimentation for 2 min on a magnetic separation rack and removal of supernatants. After air-drying of the washed beads, on-bead protein digestion with trypsin was initiated. For protein extracts of 2×10^6 bacterial cells 40 ng trypsin, and for those of 5×10^6 bacterial cells 80 ng trypsin were added to the bead solution and incubated for 18 h at 37 °C. All protein digestions were performed in 25 µL ABC-buffer (20 mmol L^{-1} ammonium bicarbonate). Digestion was stopped by adding ACN to a final ACN concentration of 95% (v/v) and subsequent incubation for 18 min with shaking at 1400 rpm Eppendorf ThermoMixer (Eppendorf, Germany). After 2 min sedimentation on a magnetic separation rack, supernatants were removed. Beads were washed with 180 µL 100% (v/v) ACN as described above and air-dried. Ten microliters 2% (m/v) DMSO were added to the dried beads to release the peptides from the beads. This step was supported by 3 min treatment in an ultrasonic bath. The peptide solution was separated from the magnetically immobilized beads and transferred into a fresh reaction tube. This tube was again placed into the magnetic separation rack and the peptide solution was transferred into a HPLC vial to avoid any transmission of beads that would disturb LC–MS/MS analysis. The peptide-containing solution was mixed with 10 µL (same amount as DMSO) twofold concentrated buffer A (4% [v/v] ACN and 0.2% [v/v] acetic acid) to obtain the required concentration for LC–MS/MS. Subsequently, samples were frozen at −80 °C until LC–MS/MS data acquisition.

Peptides were analyzed using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, MA, USA) coupled with an UltiMate 3000 nano-LC system (Thermo Fisher Scientific). Peptide separation was accomplished with the combination of a precolumn (Acclaim PepMap; Thermo Fisher Scientific) and analytical column (Accucore; Thermo Fisher Scientific). A binary gradient of buffer A (0.1% [v/v] acetic acid in HPLC-grade water [Thermo Fischer Scientific]) and buffer B (0.1% [v/v] acetic acid in ACN) and a flow rate of 300 nL min^{-1} were used. Samples were analyzed with a data dependent acquisition (DDA) method. Details are provided in Table S2, Supporting Information. Resulting raw data were processed using MaxQuant version 1.5.3.30.[23] In brief, the following settings were used: Trypsin/P as proteolytic enzyme with two missed cleavage sites allowed, as variable modifications methionine oxidation and acetylation of protein N-termini were set and a PSM and a protein FDR of 0.01 was used. Proteins were only considered identified, if two or more unique peptides were found per protein. Details are provided in Table S3, Supporting Information. *S. aureus* NCTC8325-4 proteins were searched against a database retrieved from Uniprot limited to *S. aureus* NCTC 8325 entries (release 09/2018, 2889 protein sequences included). *S. suis* proteins were searched against an NCBI database for strain P1/7_ID218494 (release 02/2018, 1833 protein sequences included). *L. pneumophila* proteins were searched against an Uniprot database limited to *L. pneumophila* Corby entries (release 01/2018, 3202 protein sequences included). Data visualization was done using RStudio version 1.1.383 with R version 3.5.1[24] and GraphPad Prism 5.0. The R packages are listed in Table S4, Supporting Information. The protein localization was analyzed using PSORTb 3.0. Data are available as Table S5, Supporting Information and a proof of principle infection experiment Table 1 and provided to MassiVE (https://massive.ucsd.edu) with the FTP download link ftp://MSV000084253@massive.ucsd.edu.

For all bacteria tested in this study, the previous species-specific CD+ISD protocol was benchmarked against a combination of species-specific cell disruption plus SDS filter-treatment with an on-bead protein digestion and peptide purification (SP3*). Applying this protocol to bacteria on filter membranes, the original SP3 protocol was adapted by adding further incubation steps with ACN after cell disruption to ensure a complete removal of SDS and a better bead-protein binding plus an additional ultrasonication step for peptide elution. For all three bacterial species tested, the SP3* protocol enabled an increased peptide and protein identification rate compared to the CD+ISD protocol (**Figure 2**; Figure S1 and Table S5, Supporting Information). For *L. pneumophila* to our knowledge only global proteome data obtained with bacterial numbers at least 50 times larger than those from infection settings, are currently available.[25,26] In the present work, on average 835 peptides that represented 303 proteins or 9% of the theoretical proteome could be identified with CD+ISD from 2×10^6 *L. pneumophila* bacteria. In contrast, about 1222 proteins (37% of the theoretical proteome) and 6040 peptides were identified with the SP3* protocol from the same number of bacteria. Thus, the number of identified proteins was increased by roughly 300% from 303 to 1222 and the number of identified peptides was increased even by >600% from

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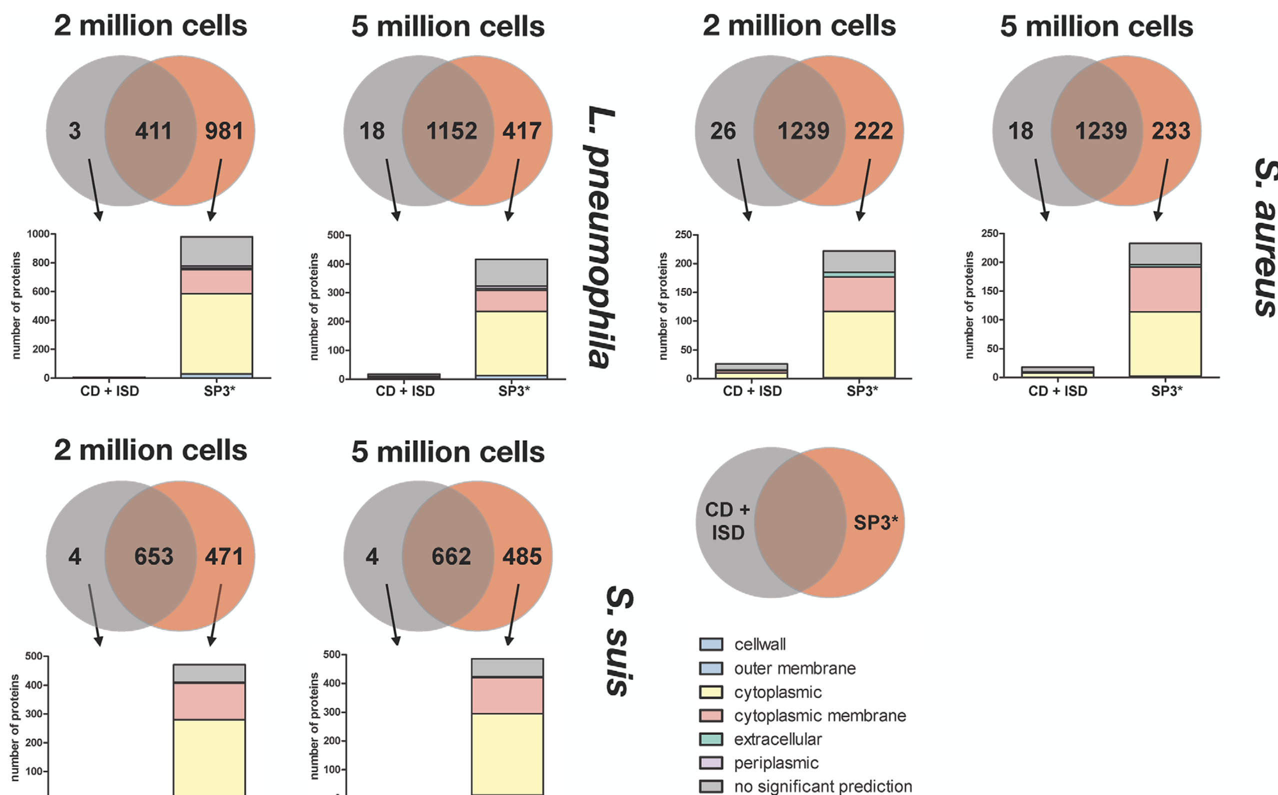

Figure 2. Comparative display of identified proteins of filter samples from *L. pneumophila*, *S. aureus*, and *S. suis* in gray number of proteins, which are only identified by in-solution digestion (CD+ISD) and in red number of proteins, which are only identified by SP3* protocol. Bar graphs below the Venn diagram, display the localization of unique detected proteins with one of those protocols.

835 to 6040. Analysis of 5×10^6 *L. pneumophila* bacteria resulted in 58% more peptides (from 5110 to 8071) and 38% more proteins (1043 to 1440) using the SP3* protocol. In comparison, Aurass et al.[25] were able to quantify 1368 different *L. pneumophila* proteins (5% less than in this study) using a fractionation approach and 20 µg protein. Thus, with the optimized SP3* protocol a similar number of proteins is amendable to quantification in less time (without fractionation) and with about 100 times fewer starting material. For 2×10^6 *S. aureus* cells, the utilization of the SP3* protocol facilitated an increase of the number of peptides and proteins by 59% (from 7118 to 11 288 peptides) and 22% (from 1142 to 1392 proteins), respectively, compared to the CD+ISD protocol. Analysis of 5×10^6 of Gram-positive *S. aureus* cells with the CD+ISD protocol led to an identification of 6883 peptides belonging to 1132 proteins. Using the newly adapted SP3* protocol, we could strongly increase peptide identifications up to 68% from 6883 to 11 545 and protein identifications up to 25% from 1132 to 1417. As a third bacterium the Gram-positive, capsule forming species *S. suis* was selected. An increase of protein identifications and especially strongly increased peptide identifications could be shown for bacterial counts as low as 2×10^6 or 5×10^6 cells, respectively. For this pathogen the number of identified peptides displayed a strong increase of 252% from 2505 to 8820 peptides, when the data of the SP3* and the CD+ISD protocol were compared. Protein identifications increased at the same time by 90% from 560 to 1063 proteins (Figure 2; Figure S1 and Table S5, Supporting Information), respectively, when the CD+ISD was substituted by the SP3* protocol. So far, there are only few global proteome studies from *S. suis* available. Recently, Yu et al. published a study where they analyzed *S. suis* proteins upon infection. These authors extracted cells from swine blood by centrifugation and digested 100 µg protein using trypsin as protease and applied a six-plex tandem mass tag (TMT) approach. In total, 1412 proteins were identified and 1147 proteins could be quantified. The data were searched against a whole *S. suis* database.[27] In comparison, our adapted SP3* protocol allowed identification and quantification of similar numbers of proteins with almost 100 times less material. Thus, not only less bacteria are required, but, also the number of animals per experiment might be reduced, especially in cases where animal samples can be pooled in order to obtain sufficient material for analysis.

In general, almost all proteins identified by the species-specific CD+ISD protocol were also covered by the adapted SP3* protocol. The proteins exclusively identified with the SP3* protocol were mostly assigned to cytoplasmic membrane, cytoplasm, or displayed no reliable prediction by PSORTb 3.0 (Figure 2). Interestingly, especially for *S. aureus* the SP3* protocol revealed identification of a distinct proportion of membrane proteins, which were not identified in the proteome prepared with the CD+ISD method probably due to limited membrane solubilization by

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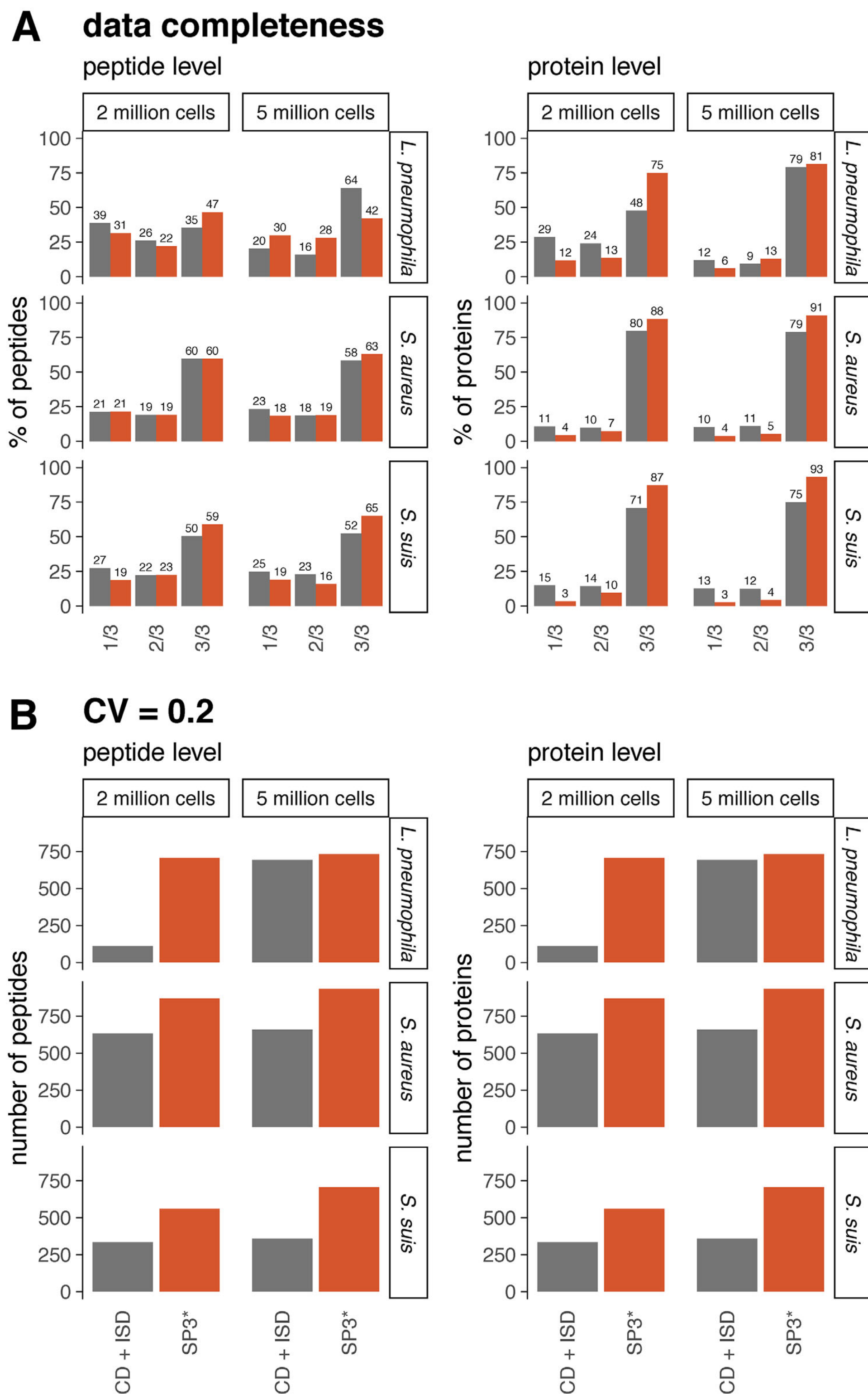

Figure 3. A) Comparison of data completeness on peptide and protein level of the bacterial specific cell disruption method (CD+ISD, gray) and a combination of the specific cell disruption combined with SDS treatment an SP3 digestion (SP3*, red). Depicted are the quantities of detected proteins or peptides in one, two, or three replicates out of three replicates. B) Number of peptides and proteins quantified with a coefficient of variation (CV) <0.2. Data obtained for *L. pneumophila* Corby, *S. aureus* NCTC8325-4, and *S. suis* strain 10 are compared.

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the original method. In brief, a strain or species-specific cell disruption in combination with treatment with 10% SDS and the bead-based SP3 protocol is advisable in order to obtain extensive peptide coverage and a high protein identification rate in MS-based proteomics with limited cell numbers (Figure 2; Table S5, Supporting Information). To evaluate the data further, the peptide count per protein was analyzed. On average counts are higher using the SP3* workflow compared to the traditional workflow. For example, for samples of *S. aureus* cells that were prepared for MS by CD+ISD on average 6.2 peptides per protein were identified and with the SP3* protocol on average 8.1 peptides per protein (Table S5, Supporting Information). In order to estimate the reproducibility of protein identifications, the detection of proteins in each of the three respective replicates was assessed. Using the SP3* protocol, the number of proteins identified in all three replicates was distinctly higher, whereas the number of proteins, which were found in only one or two of the three replicates, was much smaller. Hence, for CD+ISD a data completeness on protein level of 48–80% in three of three replicates in comparison to 75–93% for the SP3* protocol, which revealed an improved data completeness (**Figure 3**A) compared to those generated with the original protocol. In addition to a higher peptide coverage, the reproducibility plays an important role for robust quantification and can be inferred from the coefficient of variation (CV) of the peptide and protein intensities among the replicates per bacterium, cell count, and method. In samples processed with SP3* protocol the proportion of peptides and proteins with a CV of 0.2 or lower was larger (Figure 3B) compared to the data generated with the original protocol. A further advantage of the SP3 protocol is its applicability to a broad range of protein amounts. Moggridge and co-workers showed that 50 µg protein and lower amounts like 1 µg are accessible by the SP3 protocol.[17] Here, we added 10% SDS for cell lysis and an additional ultrasonication step to ensure complete recovery of bacterial proteins from the membrane filters and to disrupt disturbing molecules such as DNA or RNA. Furthermore, efficient removal of debris, detergents, and salts in the subsequent bead-based procedure enables application of high concentrations of detergents to completely disrupt cells and solubilize membranes without interfering with later MS measurements. We could show that even lower protein amounts in the range of 0.12–0.9 µg can be processed with this method (Table S6, Supporting Information). Practical advantages of the protocol include relatively simple and fast sample preparation and decreased costs compared to C_{18} ZipTip purification, which was always required for in-solution digestion. Especially for low sample amounts of the selected Gram-negative and Gram-positive bacteria with or without capsule, improvements were convincing. In an additional proof of principle infection experiment with *S. aureus*, we could further support improvements in identification rate, quantification, and data completeness (Supporting Information Proof of principle infection experiment).

Thus, the optimized workflow presented in this work can not only be applied to diverse bacteria but also to different types of samples with low bacterial amounts. This includes not only internalization experiments in cell culture, but also samples from animal experiments, human specimen, and environmental samples.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

bacterial cell disruption, internalization, proteomics, small cell numbers, SP3

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[1] D. Becker, M. Selbach, C. Rollenhagen, M. Ballmaier, T. F. Meyer, M. Mann, D. Bumann, *Nature* **2006**, *440*, 303.
[2] M. Depke, K. Surmann, P. Hildebrandt, N. Jehmlich, S. Michalik, S. E. Stanca, W. Fritzsche, U. Völker, F. Schmidt, *Cytometry A* **2014**, *85*, 140.
[3] S. Michalik, M. Depke, A. Murr, M. Gesell Salazar, U. Kusebauch, Z. Sun, T. C. Meyer, K. Surmann, H. Pförtner, P. Hildebrandt, S. Weiss, L. M. P. Medina, M. Gutjahr, E. Hammer, D. Becher, T. Pribyl, S. Hammerschmidt, E. W. Deutsch, S. L. Bader, M. Hecker, R. L. Moritz, U. Mäder, U. Völker, F. Schmidt, *Sci. Rep.* **2017**, *7*, 9718.
[4] S. M. Twine, N. C. S. Mykytczuk, M. D. Petit, H. Shen, A. Sjöstedtb, J. W. Conlana, J. F. Kellya, *Biochem. Biophys. Res. Commun.* **2006**, *345*, 1621.
[5] Q. Xia, T. Wang, F. Taub, Y. Park, C. A. Capestany, R. J. Lamont, M. Hackett Dr, *Proteomics* **2007**, *7*, 4323.
[6] Y. Abu Kwaik, B. I. Eisenstein, N. C. Engleberg, *Infect. Immun.* **1993**, *61*, 1320.
[7] D. Benndorf, C. Vogt, N. Jehmlich, Y. Schmidt, H. Thomas, G. Woffendin, A. Shevchenko, H.-H. Richnow, M. von Bergen, *Biodegradation* **2009**, *20*, 737.
[8] N. Jehmlich, T. Hübschmann, M. Gesell Salazar, U. Völker, D. Benndorf, S. Müller, M. von Bergen, F. Schmidt, *Appl. Microbiol. Biotechnol.* **2010**, *88*, 575.
[9] C. Wiacek, S. Müller, D. Benndorf, *Proteomics* **2006**, *6*, 5983.
[10] J. Liu, J. Li, L. Feng, H. Cao, Z. Cui, *J. Microbiol.* **2010**, *48*, 728.
[11] T. Nishino, Y. Matsuda, Y. Yamazaki, *Heliyon* **2018**, *4*, e00597.
[12] Z. Fu, S. Rogelj, T. L. Kieft, *Int. J. Food Microbiol.* **2005**, *99*, 47.

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[13] J.-H. Kim, J. G. Yoo, J.-S. Ham, M.-H. Oh, *Korean J. Food Sci. Anim. Resour.* **2018**, *38*, 727.
[14] C. Trötschel, A. Poetsch, *Proteomics* **2015**, *15*, 915.
[15] K. R. Ludwig, M. M. Schroll, A. B. Hummon, *J. Proteome Res.* **2018**, *17*, 2480.
[16] C. S. Hughes, S. Foehr, D. A. Garfield, E. E. Furlong, L. M. Steinmetz, J. Krijgsveld *Mol. Syst. Biol.* **2014**, *10*, 757.
[17] S. Moggridge, P. H. Sorensen, G. B. Morin, C. S. Hughes, *J. Proteome Res.* **2018**, *17*, 1730.
[18] C. S. Hughes, S. Moggridge, T. Müller, P. H. Sorensen, G. B. Morin, J. Krijgsveld, *Nat. Protoc.* **2019**, *14*, 68.
[19] M. Sielaff, J. Kuharev, T. Bohn, J. Hahlbrock, T. Bopp, S. Tenzer, U. Distler, *J. Proteome Res.* **2017**, *16*, 4060.
[20] S. Herbert, A.-K. Ziebandt, K. Ohlsen, T. Schäfer, M. Hecker, D. Albrecht, R. Novick, F. Götz, *Infect. Immun.* **2010**, *78*, 2877.
[21] P. Hildebrandt, K. Surmann, M. G. Salazar, N. Normann, U. Völker, F. Schmidt, *Cytometry A* **2016**, *89*, 932.
[22] L. M. Palma Medina, A.-K. Becker, S. Michalik, H. Yedavally, E. J. M. Raineri, P. Hildebrandt, M. G. Salazar, K. Surmann, H. Pförtner, S. A. Mekonnen, A. Salvati, L. Kaderali, J. M. van Dijl, U. Völker, *Mol. Cell. Proteomics* **2019**, *18*, 892.
[23] J. Cox, M. Mann, *Nat. Biotechnol.* **2008**, *26*, 1367.
[24] R Core Team, *R 3.5.2*, R Foundation for Statistical Computing, Vienna, Austria **2018**.
[25] P. Aurass, T. Gerlach, D. Becher, B. Voigt, S. Karste, J. Bernhardt, K. Riedel, M. Hecker, A. Flieger, *Mol. Cell. Proteomics* **2016**, *15*, 177.
[26] W. M. Bruckert, Y. Abu Kwaik, *J. Proteome Res.* **2015**, *14*, 236.
[27] Y. Yu, Y. Qian, D. Du, Q. Li, C. Xu, H. Liu, M. Chen, H. Yao, C. Lu, W. Zhang, *J. Proteomics* **2018**, *180*, 41.

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