Proteome dynamics during sporulation and heat resistance in Bacillus subtilis spores

Tu, Z.

Publication date
2021

Chapter 5

The influence of sample preparation on the composition of the cellular and spore proteome of *Bacillus subtilis*

Zhiwei Tu [1,2], Winfried Rosenboom [2], Henk L. Dekker [2], Bhagyashree N. Swarge [1,2], Stanley Brul [1] and Gertjan Kramer [2]

[1] Laboratory for Molecular Biology and Microbial Food Safety, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands; Z.Tu@uva.nl (Z.T.); B.N.Swarge@uva.nl (B.N.S.); s.brul@uva.nl (S.B.).

[2] Laboratory for Mass Spectrometry of Biomolecules, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands; H.L.Dekker@uva.nl (H.L.D); W.Roseboom@uva.nl (W.R); g.kramer@uva.nl (G.K.).

Abstract

Bacterial endospores are able to survive harsh environments commonly lethal to their cellular counterparts. The spore resistance capacity relies highly on multilayered coat structures. To detect the complete spore proteome, we previously developed the "one-Pot" sample processing method. It uses 6 M urea to facilitate protein extraction. Meanwhile, a single-pot, solid phase-enhanced sample-preparation (SP3) has emerged which uses sodium dodecyl sulfate (SDS) for protein extraction. In this study, we compare the utility of SP3 protein and peptide clean-up for the analysis of *B. subtilis* spores and cells in comparison to our established "one-Pot" method. We show that SP3 protein clean-up identifies the maximum numbers of peptides (8400 in cells and 6400 in spores) and proteins (more than 1200 in both cells and spores). Also, SP3 protein clean-up is capable of qualitatively and quantitively identifying more membrane associated proteins. In conclusion, SP3 protein clean-up can replace the "one-pot" method in proteomic studies of cells and spores of *B. subtilis*.

1. Introduction

Bacterial endospores are one of the toughest forms of life on earth. Efficient elimination of spores still challenges the food processing and healthcare industries[1,2]. The extreme resistance of spores is due in part to the multilayered proteinaceous spore coat[3]. To facilitate studying the molecular make-up of the spore coat, a "gel-free" protocol that specifically targeted the insoluble coat proteins identified hitherto unknown spore coat proteins[4]. The proteome of the spore inner membrane, which is important in both resistance to harsh conditions and in germination, has also been analyzed by mass spectrometry[5]. In these[4,5], as well as in other studies, SDS is used to increase protein extraction and is removed by extensive buffer exchange or washing.

The "one-pot" sample processing was designed to qualitatively and quantitively study the proteome of intact spores with no extra steps to remove detergents[6]. Use of SDS was avoided because of its deleterious effects on reversed phase chromatography and ionization suppression during electrospray ionization[7,8]. Instead, urea was employed to improve solubilization of spore proteins and is easily removed prior to liquid chromatography mass spectrometry (LCMS) analysis. A drawback to using urea for protein solubilization in proteomic studies is its potential to cause carbamylation at lysine and arginine residues, negatively affecting protease digestion, protein identification, and the study of *in vivo* protein carbamylation. Special care has to be taken not to overheat the urea containing extraction buffer during sample disruption to limit isocyanate formation. Use of ammonium containing buffers in conjunction with urea also limits unwanted protein carbamylation[9].

Recently, a single-pot, solid phase-enhanced sample-preparation (SP3) method has been described which is compatible with a wide range of protein solubilizing reagents, such as SDS, and can process samples in a single vessel[10]. It can both prepare mass spectrometry ready peptides from a digest as well as cleaned-up protein samples prior to enzymatic digestion. In this chapter, we test this SP3 peptide and protein clean-up method on *B. subtilis* vegetative cells and spores compared to the gold standard "one pot" method for analysis of bacterial spores and vegetative cells.

Equal numbers of cells and spores of *B. subtilis* PY79 were processed using the SP3 peptide, protein clean-up and "one-pot" methods. The processed samples were analyzed using LC-MS (liquid chromatography–mass spectrometry), and numbers of identified peptides and proteins were compared. In assessing the reproducibility of the various methods, coefficients of variation (CV) of replicates were calculated. Furthermore, we looked at whether certain types of proteins were over or underrepresented in the different sample processing techniques by using gene ontology cellular component (GOCC) analysis. The results show that SP3 protein clean-up is capable of identifying the maximum number of peptides, around 8400 peptides in cells and 6400 peptides in spores, which come from more than 1200 proteins. Moreover, SP3 protein clean-up could qualitatively and quantitively identify more membrane proteins. In terms of reproducibility, SP3 peptide clean-up gave the minimum median CVs in cells and "one-pot" showed the minimum median CVs in spores. Consequently, SP3 protein clean-up outperforms "one-pot" in identification and quantification of spore and cellular proteins of *B. subtilis* and could be considered the new gold standard in bacterial and spore proteomics.

2. Results

2.1. Identification of peptides and proteins of B. subtilis cells and spores

Spore and cellular samples were subjected to SP3 peptide & protein clean-up and "one-pot" sample processing methods (**Figure 1**), and the tryptic peptides were analyzed by LC-MS. Data from cells and spores were analyzed independently. In both cells and spores, SP3 peptide clean-up is only able to identify half number of the peptides identified in SP3 protein clean-up (average 8400 in cells and 6400 in spores) (**Figure 2**). The "one-pot" method identifies a similar but smaller number of peptides than the SP3 protein clean-up. At the protein level, SP3 peptide clean-up identifies ~ 1000 proteins in both cells and spores, whereas SP3 protein clean-up and "one-pot" identify more than 1200 proteins (**Figure 2**).

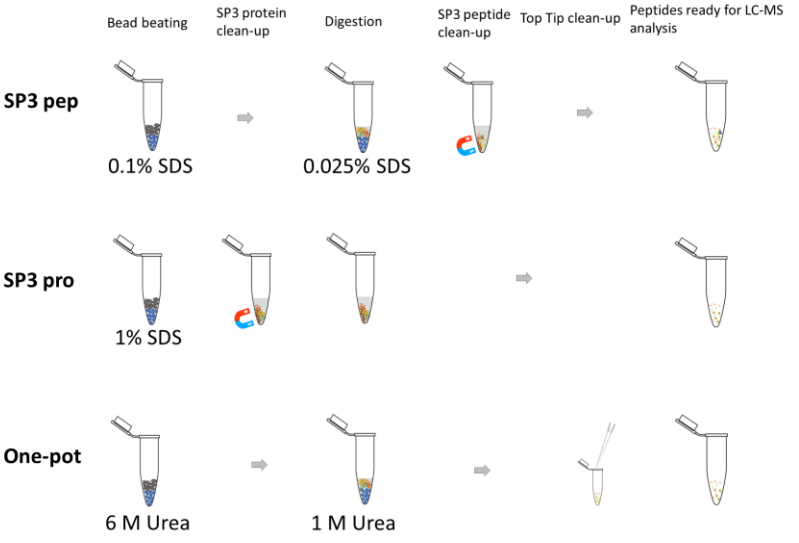

Figure 1. Schematic diagram of SP3 peptide (SP3 pep) & protein (SP3 pro) clean-up and "one-pot" methods. Following bead beating, SDS in SP3 pep, and urea in "one-pot" was diluted to the stated concentrations using the digestion buffer. Trypsin was added to the diluted samples to digest proteins into peptides. Peptides were purified from the digest by SP3 peptide clean-up aided by a magnet, and TopTip clean-up separately. In SP3 pro, proteins were first purified form the lysate using SP3 protein clean-up aided by a magnet, and then digested into peptides.

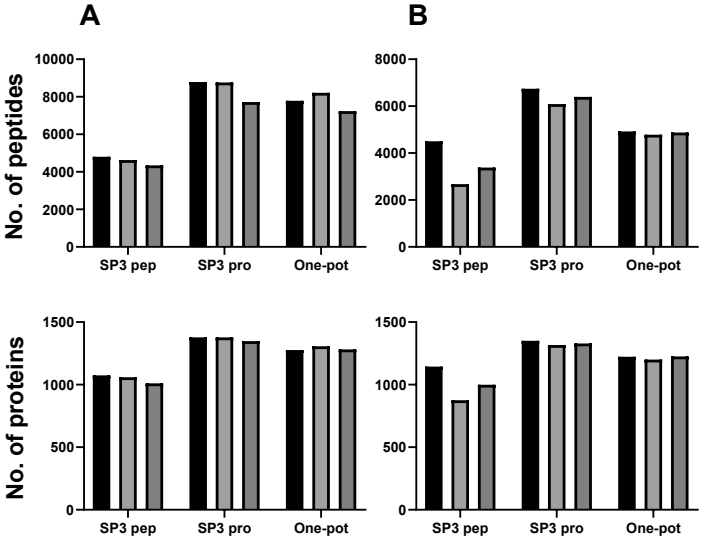

Figure 2. Number of peptides & proteins identified in *B. subtilis* cells (A) and spores (B). Three biological replicates were performed for each method. Each bar for a method indicates the number of identified peptides or proteins.

Identified proteins by three methods were compared. 986 proteins in cells and 860 proteins in spores were identified in all three methods (**Figure 3A**) The additional 7 proteins in cells and 3 proteins in spores specifically identified by the SP3 peptide clean-up were quite few in number compared to the additional 122 cellular and 173 spore proteins identified in the SP3 protein clean-up and the 45 cellular and 157 spore proteins identified in the "one-pot" method. In terms of proteins identified by two of the three methods, the SP3 protein clean-up and "one-pot" identified the largest numbers of proteins, 234 in cells and 189 in spores. The identified proteins were subjected to GOCC analysis. The top 10 categories are shown in **Figure 3B**. In cells, the SP3 protein clean-up gave similar results compared to the "one-pot" method in the numbers of proteins belonging to the GOCC categories displayed. However, the SP3 peptide clean-up leads to a lower number of proteins in the plasma membrane and integral component of membrane categories. In spores, SP3 protein clean-up turned out to be able to identify more proteins from plasma membrane and integral component of membrane categories. The SP3 peptide clean-up identified similar but smaller numbers of proteins in GOCC categories compared to the "one-pot" method.

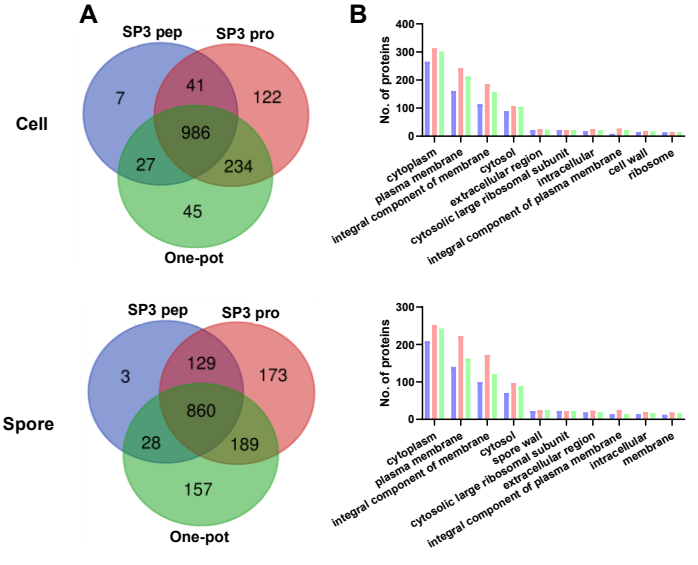

Figure 3. Comparison of proteins identified by the SP3 peptide and protein clean-up and "one-pot" methods. (A) Venn diagram of the identified proteins from the SP3 peptide & protein clean-up and "one-pot" sample processing methods. (B) Number of proteins clustered to GOCC categories. Colors of the bars correspond to those in (A).

Germinant receptors (GRs) are key components in spore germination and their levels are low in spores[11]. Both the SP3 peptide and protein clean-up were capable of identifying GRs and other germination proteins (**Table 1**). In total, 85, 184 and 151 peptides of germination proteins were identified by the SP3 peptide & protein clean-up and "one-pot" methods, respectively. A higher number of unique peptides was identified by the SP3 protein clean-up for some proteins, such as GerAA, GerBA, GerKC, SpoVAEa and SpoVAF, and some proteins have more unique peptides identified by the "one-pot" method, for example, GerPC and CwlJ. Most germination proteins known to be localized in the inner membrane were identified by all three methods. Spore coat proteins identified are listed in **Table 2**. The coverage of coat proteins identified by the SP3 protein clean-up and "one-pot" methods are quite similar, and lead to identification of similar numbers of peptides of coat proteins (881 and 827 peptides respectively). However, the SP3 peptide clean-up identified only 506 peptides from coat proteins.

Table 1, germination proteins identified in this study and in the spore inner membrane[a]

Gene	Total no. of unique peptides			Identified in this study[b]			identified in inner membrane[b,c]
	SP3 pep	SP3 pro	"One-pot"	SP3 pep	SP3 pro	"One-pot"	
gerAA	4	11	9	**	**	**	NA
gerAB	0	0	0	NA	NA	NA	*
gerAC	4	8	8	**	**	**	NA
gerBA	1	7	4	*	**	**	**
gerBC	1	3	3	*	**	**	*
gerKA	8	8	5	**	**	**	**
gerKB	0	2	0	NA	**	NA	**
gerKC	7	24	4	**	**	**	**
gerPA	0	2	3	NA	**	**	NA
gerPB	0	0	3	NA	NA	**	NA
gerPC	1	1	6	*	*	**	NA

gerPF	0	0	3	NA	NA	**	NA
spoVAA	0	3	0	NA	**	NA	**
spoVAC	0	3	3	NA	**	**	**
spoVAD	17	24	30	**	**	**	**
spoVAEa	3	9	3	**	**	**	**
spoVAF	9	22	11	**	**	**	**
cwlJ	5	8	12	**	**	**	NA
sleB	18	26	20	**	**	**	**

a, the protein list is acquired from[12]. b, *, identified in one replicate; **, identified in at least two replicates. c, acquired from[5]. NA, not identified.

Table 2, Spore coat proteins identified in this study[a]

Gene	Total no. of unique peptides			Identified in this study[b]		
	SP3 pep	SP3 pro	"One-pot"	SP3 pep	SP3 pro	"One-pot"
spoIVA	19	29	27	**	**	**
spoVID	0	0	3	NA	NA	**
spoVM	1	6	0	*	**	NA
yaaH	47	50	38	**	**	**
yuzC	8	7	6	**	**	**
cotE	17	17	24	**	**	**
cotM	0	3	0	NA	**	NA
cotO	0	3	2	NA	**	**
yhjR	15	23	14	**	**	**
yncD	5	18	12	**	**	**
cotZ	0	3	12	NA	**	**
cwlJ	5	9	12	**	**	**
yisY	9	27	18	**	**	**
yutH	3	6	3	**	**	**
yybI	7	15	11	**	**	**
cotA	34	59	53	**	**	**
cotB	11	34	31	**	**	**
cotG	2	8	8	**	**	**
cotP	0	0	3	NA	NA	**
cotQ	9	29	37	**	**	**
cotS	12	21	34	**	**	**
cotW	6	12	5	**	**	**
lipC	19	31	12	**	**	**
oxdD	12	26	18	**	**	**
tgl	15	21	9	**	**	**
yjqC	17	19	22	**	**	**
yjzB	2	3	0	**	**	NA
yppG	0	0	5	NA	NA	**
ytxO	7	15	7	**	**	**
yxeE	10	11	16	**	**	**

cotD	0	0	2	NA	NA	**
cotU	0	0	10	NA	*	**
cgeA	8	11	6	**	**	**
cgeC	0	2	3	NA	**	**
cotC	10	18	10	**	**	**
cotF	11	16	21	**	**	**
cotH	6	17	13	**	**	**
cotI	16	23	24	**	**	**
cotJA	9	11	9	**	**	**
cotJB	7	2	13	**	**	**
cotJC	18	24	23	**	**	**
cotR	9	23	21	**	**	**
cotSA	23	35	29	**	**	**
cotX	6	6	11	**	**	**
cotY	7	11	25	**	**	**
gerQ	8	9	18	**	**	**
ldt	4	12	3	**	**	**
safA	13	23	24	**	**	**
spsB	0	3	3	NA	**	**
yabG	3	9	10	**	**	**
ydhD	11	27	24	**	**	**
ygaK	10	28	32	**	**	**
yhbB	4	16	17	**	**	**
yheC	0	7	0	NA	**	NA
yjdH	6	8	9	**	**	**
ykvP	2	7	12	*	**	**
yodI	5	5	6	**	**	**
ypeP	5	8	6	**	**	**
yqfT	13	15	1	**	**	*

a, the protein list is acquired from *Subti*Wiki[13]. b, *, identified in one replicate; **, identified in at least two replicates. NA, not identified.

2.2. Quantification of peptides and proteins of B. subtilis cells and spores

We assessed the reproducibility of each method by calculating the coefficient of variation (CV) for the LFQ intensities of peptides and proteins for the three sample preparation methods (**Figure 4**). In cells, SP3 peptide clean-up shows the minimum median CVs - 0.18 on the peptide level and 0.15 on the protein level. However, in spores, the “one-pot” method shows the minimum median CVs - 0.17 on the peptide level and 0.16 on the protein level.

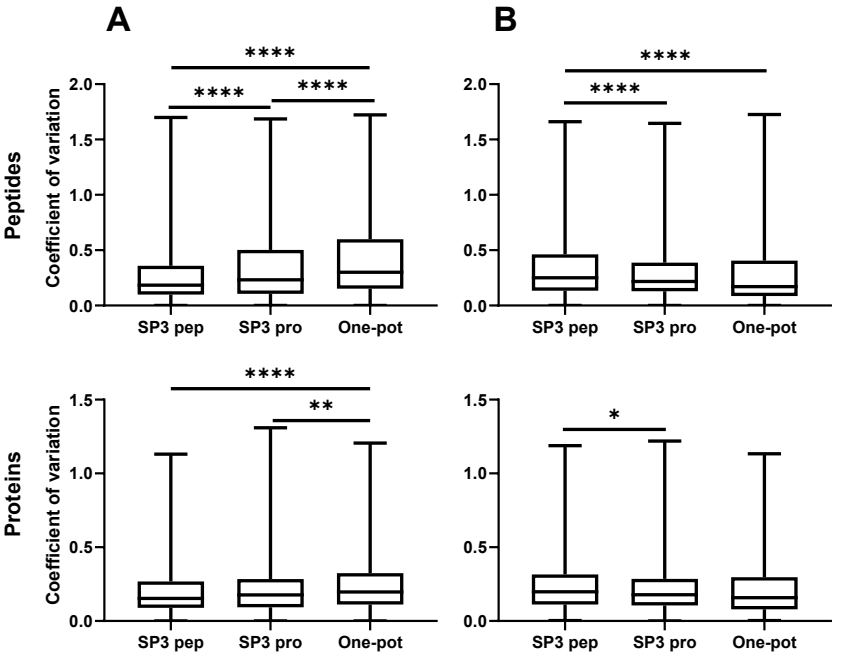

Figure 4. Box plots of CVs of the peptides & proteins quantified in at least 2 of 3 replicates in *B. subtilis* cells (A) and spores (B). Statistical significance was determined by one-way analysis of variance (ANOVA). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

Proteins quantified in all three methods are classified in different clusters in K-means cluster analysis (**Figure 5A**). In cells, the SP3 protein clean-up extracted smaller amounts of proteins in cluster K1, and higher amounts of proteins in cluster K2, than the SP3 peptide clean-up and "one-pot" methods. In spores, the SP3 protein clean-up extracted lower amounts of proteins in cluster K1, and higher amounts of proteins in cluster K3, than the SP3 peptide clean-up and "one-pot" methods. Furthermore, the SP3 peptide & protein clean-up processing methods extracted lower amounts of proteins in cluster K2 than the "one-pot" processing method. In the GOCC analysis (**Figure 5B**), the cellular K2 and spore K3 clusters which have an increased amount of protein extracted, contain a larger proportion of proteins from the plasma membrane and integral components of the membrane compared to the other clusters. In addition, spore cluster K2 has a relatively higher fraction of spore coat proteins and spore

cluster K3 has a relatively higher fraction of cytosolic proteins.

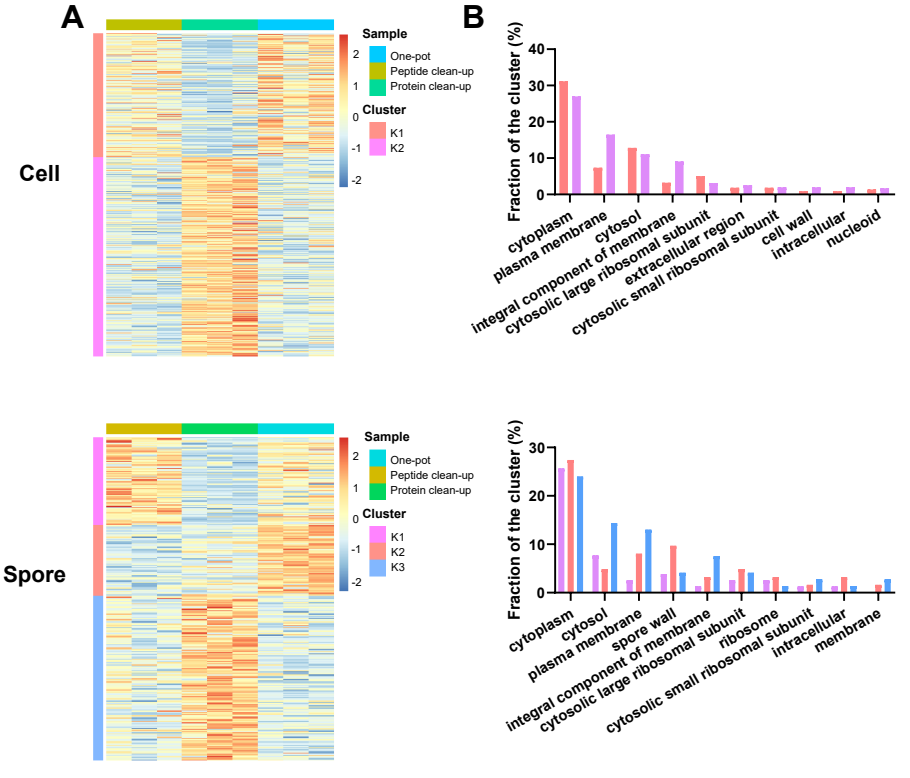

Figure 5. Comparison of proteins quantification by SP3 peptide & protein clean-up and One-pot methods. (A) Heat map of the quantified proteins. Proteins from cells and spores are clustered in 2 (K1 and K2) and 3 (K1, K2 and K3) clusters in K-means cluster analysis. (B) Bar graphs of fractions of the cluster classified to GOCC categories. The top 10 categories are shown in the graphs. Colors of the bars correspond to the clusters in (A).

3. Discussion

The "one-pot" sample processing method has been developed in the lab to deal with the complexity of the bacterial spore proteome. It has been successfully applied in the study of spores (chapter 4 and[6,14]), spore germination[15,16] and spore formation (sporulation, chapter 3). However, it falls short in compatibility with different protein solubilizing reagents, for example, SDS, one of the most popular laboratory reagents used in the sample protein

extraction. What's more, sample overheating may give rise to problems caused by protein carbamylation. The SP3-based workflow has the advantage of compatibility with a wide range of protein solubilization reagents and working with low sample amounts in the sub microgram range[17]. In this chapter we demonstrated the applicability of an SP3-based method to proteomic analysis of bacterial spores and vegetative cells. With *B. subtilis* vegetative cells and spores, the SP3 protein method identified the largest number of peptides. This could be caused by the presence of SDS in SP3 peptide clean-up and urea in the ''one-pot'' method during digestion, affecting the activity of trypsin. This could decrease the overall efficiency of protein digestion and reduce the peptide identification rate, even though most of the activity is reported to be retained in a solution containing 0.1% (w/v) SDS or 1 M urea according to the product information and[18]. Moreover, 0.1% SDS may be not strong enough to solubilize all membrane proteins during the SP3 peptide clean-up workflow. The CVs among three methods are distributed differently at both peptide and protein level. However, the median CVs between three methods are similar and well within the expected range for label free LCMS analysis. Furthermore, an automated sample preparation for proteomics implemented with the SP3 protein clean-up (autoSP3) has been introduced[19]. With autoSP3, reproducibility among replicates is further improved.

The "one-pot" sample processing method should be avoided when studying *in vivo* carbamylation as it can cause sample preparation induced artifacts as discussed in the introduction. In GOCC analysis, a larger number of proteins from the plasma membrane and integral component of membrane categories are qualitatively and quantitively identified in spores and cells using the SP3 protein clean-up. This underscores the advantages of using SDS for protein extraction to cover membrane proteins better in bacterial cell and spore proteomics. Two important aspects for studies into spore biology are spore germination and spore resistance properties. The methods used to study spore proteomes must be capable of identifying major germination and coat proteins. In this study, all three methods showed similar coverage on the identification of germination and coat proteins. However, the SP3 protein clean-up identified 184 unique peptides of germination proteins which are far more than that

identified by the SP3 peptide clean-up and "one-pot" methods. Furthermore, SP3 protein clean-up identified 881 peptides for coat proteins which is far more than that identified by the SP3 peptide clean-up and a little more than the "one-pot" method.

4. Conclusion

Overall, we demonstrated the applicability of the SP3 protein clean-up to bacterial spores and cells. This method outperformed the "one-pot" method in identification and quantification of membrane proteins and the total number of identified peptides and proteins, and it could replace the "one-pot" method for processing spores and cells of *B. subtilis* in proteomic studies. However, identification of integral membrane proteins such as B subunits of GRs and some SpoVA proteins remains challenging.

5. Materials and Methods

5.1. Strain and sporulation

Bacillus subtilis PY79 was used in this study. Sporulation of the vegetative cells was done following the protocol in[4]. Briefly, cells from a single colony were incubated in Lysogeny broth (LB) medium at 37°C and 200 rpm until exponential phase (OD_{600} 0.3-0.5) was reached. 1 ml of culture was transferred to 20 ml of MOPS-buffered medium to continue growing at 37°C[1]. When the exponential phase was reached again, 5 ml culture were transferred to 500 ml MOPS-buffered medium to sporulate at 37°C for 96 h. Spores were purified from the remaining cells using Histodenz (Sigma-Aldrich, St. Louis, Missouri, USA)[20]. Vegetative cells were harvested from LB medium at exponential phase. Three biological replicate samples were collected for spores and cells. Every replicate was divided into three portions and they were processed by the SP3 protein and peptide clean-up and the "one-pot" methods.

5.2. SP3 protein clean-up and digestion for mass spectrometry

The Sample lysis protocol was adapted from the "one-Pot" method[6]. Cells or spores were suspended in a 200 ul lysis buffer containing 1% SDS (sodium dodecyl sulfate), 10 mM TCEP (Tris (2-carboxyethyl) phosphine hydrochloride), 30 mM CAA (2-Chloroacetamide) and 100 mM ammonium

bicarbonate in a 2 ml screw-top tube with an O-ring cap (Sigma-Aldrich, St. Louis, Missouri, USA). Zirconium-silica beads (0.1 mm, BioSpec Products, Bartlesville, OK, USA) were added to the screw-top tube until the beads reached the top surface of the buffer. Samples were disrupted using a Precellys 24 homogenizer (Bertin Technologies, Aix en Provence, France). The disruption program includes 7 rounds bead beating at 6000 rpm with 20 s for each round and 60 s pause between each round. Samples were placed on ice to lower the temperature between the disruption runs. Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (CAT NO. 23250) according to the manufacturer's instruction. The SP3 protein extraction protocol is adapted from[10,17,21]. Briefly, carboxylate-modified magnetic beads (Sera-Mag™ Magnetic carboxylate modified particles (Hydrophilic), CAT NO. 24152105050250, and Sera-Mag™ Magnetic carboxylate modified particles (Hydrophobic), CAT NO. 44152105050250) can covalently couple peptides or proteins in a neutral condition and decouple them in an acidic condition. Two types of beads (hydrophilic and hydrophobic) were mixed 1:1 (v/v), washed and resuspended in water at a concentration of 20 ug/ul. 50 µl sample lysates containing ~ 20 ug of protein were mixed with 2 ul magnetic bead mixture in a 200 ul PCR tube. Acetonitrile was added to a final percentage of 50% (v/v). After incubation for 18 min at room temperature, the tube was placed on a magnetic rack for 2 min. The beads which had coupled proteins settled to the bottom of the tube. Supernatant was discarded and the beads were washed with 200 ul 70% ethanol two times. After that, the beads were washed with 200 ul acetonitrile again and air dried. 10 ul digestion buffer containing 100 mM ammonium bicarbonate was used to resuspend beads. 400 ng trypsin was added to digest proteins overnight at 37°C. The peptides were decoupled from the beads by acidifying the supernatant with formic acid to a final concentration of 1%. Transfer the supernatant to a new tube and the sample was ready to be analyzed by LC-MS.

5.3. SP3 peptide clean-up and digestion for mass spectrometry

The sample lysis protocol was the same as in the SP3 protein clean-up, but the SDS concentration was 0.1%. Following the bead beating and BCA protein assay, 600 ul of the lysis buffer containing 100 mM ammonium bicarbonate was added to dilute SDS to 0.025%. Trypsin with a 1:50 enzyme to

substrate ratio was added and the mix digested overnight at 37°C. Peptide clean-up was accomplished using the same carboxylate-modified paramagnetic beads described in the SP3 protein clean-up. Sample lysates containing 20 ug digest were taken to mix with 2 ul magnetic bead mixture. Acetonitrile was added to obtain a final percentage of 95%. After 18 minutes incubation at room temperature, the tube was placed on a magnetic rack for 2 minutes to let the beads to settle. The supernatant was discarded, and the beads were washed two times with acetonitrile. Air dried beads were reconstituted with 20 ul 0.1% formic acid and sonicated for 5 minutes. Supernatants were transferred to new tubes and the tryptic peptides were ready for LCMS analysis.

5.4. "One-pot" sample processing and digestion for mass spectrometry

Samples were processed and digested following the "one-pot" method[6]. Cells or spores were suspended in a 200 ul lysis buffer containing 6 M urea, 10 mM TCEP, 30 mM CAA and 50 mM ammonium bicarbonate. Bead beating and BCA protein assay was done as described in SP3 protein clean-up. 1 ml lysis buffer containing 50 mM ammonium bicarbonate was added to dilute urea to 1 M, and trypsin with 1:50 enzyme to substrate ratio was added to digest overnight at 37°C. The digestion reaction was quenched with the addition of formic acid to pH < 4. Peptide containing supernatant was collected by centrifuging for 15 min at 15000 rpm. Peptide clean-up was done using C18 reversed-phase TT2 Top-Tips (Glygen) according to the manufacturer's instruction.

5.5. LC-MS/MS

Samples were reconstituted in 0.1% formic acid in water and 200 ng equivalent was injected by a Ultimate 3000 RSLCnano UHPLC system (Thermo Scientific, Germeringen, Germany) onto a 75um x 250 mm analytical column (C18, 1.6 um particle size, Aurora, Ionopticks, Australia) kept at 50°C at 400 nl/min for 15 minutes in 3% solvent B before being separated by a multi-step gradient (Solvent A: 0.1% formic acid in water, Solvent B: 0.1% formic acid in acetonitrile) to 5% B at 16 min, 17% B at 38 min, 25% B at 43 min, 34% B at 46 min, 99% B at 47 min held until 54 min returning to initial conditions at 55 min equilibrating until 80 min.

Eluting peptides were sprayed by the emitter coupled to the column into a captive spray source (Bruker, Bremen Germany) with a capillary voltage of 1.5 kV, a source gas flow of 3 L/min of pure nitrogen and a dry temperature setting of 180°C, attached to a timsTOF pro (Bruker, Bremen Germany) trapped ion mobility, quadrupole, time of flight mass spectrometer. The timsTOF was operated in PASEF mode of acquisition. The TOF scan range was 100-1700 m/z and a tims range of 0.6-1.6 V.s/cm^2. In PASEF mode a filter was applied to the m/z and ion mobility plane to select features most likely representing peptide precursors, the quad isolation width was 2 Th at 700 m/z and 3 Th at 800 m/z, and the collision energy was ramped from 20-59 eV over the tims scan range to generate fragmentation spectra. A total of 10 PASEF MS/MS scans scheduled with a total cycle time of 1.16 seconds, scheduling target intensity 2e4 and intensity threshold of 2.5e3 and a charge state range of 0-5 were used. Active exclusion was on (release after 0.4 min), reconsidering precursors if ratio current/previous intensity >4.

5.6. Data processing and bioinformatics

Collected LCMS data from cells and spores were analyzed using MaxQuant (Version 1.6.14)[22] in two independent analyses. The data were searched against a UniProt proteome database (proteome ID UP000001570)[23]. The instrument type was Bruker TIMS. Digestion enzyme was Trypsin/P with a maximum of 2 missed cleavages. LFQ method was selected for the label-free quantification. Other parameters were set as the default values.

The output identified peptides and proteins and their LFQ quantitation were extracted from the files peptides.txt and proteinGroup.txt. The potential contaminant and identified reverse peptides and proteins were removed from the results. Identified peptides and proteins were counted and compared. Proteins identified at least two times in one of three methods were analyzed further to investigate their proteome coverages. To compare the reproducibility of the three methods, coefficient of variations (CVs) of the peptides and proteins quantified in more than two replicates were calculated. Proteins quantified in three replicates were used to compare the protein recovery efficiency between the methods. K-means clustering was used to classify the proteins in different

groups. Functional annotation of identified and quantified proteins from the three methods was carried out with the help of tools available at DAVID bioinformatics resources[24].

Acknowledgements

We thank Prof. dr. Peter Setlow for critical reading of the manuscript.

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