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DOI
10.1021/acs.jproteome.5b00976

Publication date
2016

Document Version
Final published version

Published in
Journal of Proteome Research

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Citation for published version (APA):

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Download date: 30 Jul 2023
**Bacillus subtilis** Spore Inner Membrane Proteome

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**Supporting Information**

**ABSTRACT:** The endospore is the dormant form of *Bacillus subtilis* and many other Firmicutes. By sporulation, these spore formers can survive very harsh physical and chemical conditions. Yet, they need to go through germination to return to their growing form. The spore inner membrane (IM) has been shown to play an essential role in triggering the initiation of germination. In this study, we isolated the IM of bacterial spores, in parallel with the isolation of the membrane of vegetative cells. With the use of GeLC–MS/MS, over 900 proteins were identified from the *B. subtilis* spore IM preparations. By bioinformatics-based membrane protein predictions, ca. one-third could be predicted to be membrane-localized. A large number of unique proteins as well as proteins common to the two membrane proteomes were identified. In addition to previously known IM proteins, a number of IM proteins were newly identified, at least some of which are likely to provide new insights into IM physiology, unveiling proteins putatively involved in spore germination machinery and hence putative germination inhibition targets.

**KEYWORDS:** Bacillus subtilis, spore inner membrane, mass spectrometry, proteome

**INTRODUCTION**

Spores of *Bacillus* and *Clostridium* species are dormant, metabolically inert structures formed particularly under nutrient starvation conditions. In their dormant form, spores are resistant to a variety of environmental insults including heat, desiccation, pressure, radiation, and toxic chemicals. Yet, the spores can detect nutrients in their surroundings and respond by triggering the resurgence of metabolism and growth via a process called germination. However, upon germination, spores lose their stress-resistant phenotype. Spore resistance and dormancy are attributed to the spore components and their structure (Figure 1). Exosporium is the outermost layer in spores of some species (but not *Bacillus subtilis*), followed by a proteinaceous coat that can be subdivided into crust, outer coat, inner coat, and basement. The outer membrane (OM) is beneath the coat layer and is sometimes described as a permeability barrier, but it is also often seen as being only a vestigial structure. The OM covers the cortex peptidoglycan layer and then the germ cell wall. The peptidoglycan of the germ cell wall and growing cell wall are identical; however, the cortex peptidoglycan has muramic acid-δ-lactam residues, which are unique to spores. Under the germ cell wall is the inner spore membrane (IM), and most of the proteins known to be involved in spore germination are present in this spore compartment. Finally, the core of the spore is where the spores’ DNA, ribosomes, and most enzymes are stored. The core possesses low water content (25–50% of wet weight) and spore-specific pyridine-2,6-dicarboxylic acid, i.e., dipicolinic acid (DPA; ~10% of total spore dry weight) chelated with divalent cations, especially to Ca²⁺.

The IM is considered to play an important role in the resistance properties of spores. This membrane is impermeable to small hydrophilic molecules unless they pass through appropriate membrane-localized small molecule transporter proteins. The IM protects the spore core and becomes the plasma membrane of the outgrown cell after germination. The OM and IM differ from each other in their lipid compositions, and the IM is the major barrier to the passage of potentially harmful chemicals into the spore core. Lipids in the IM are largely immobile, although lipid mobility is regained upon germination. The protein and lipid components of the IM and the forces acting on this membrane due to the core or cortex could, together, play some role in determining the membrane’s properties. The IM of dormant spores exists in a compressed form; in the absence of lipid synthesis, its surface area is said to...
expands approximately 2-fold after spore germination is completed. In previous studies, it was observed that damage to the IM plays an important role in spore killing by heat and oxidizing agents. High temperatures can cause disruption of the IM, releasing DPA from the spore core. Oxidizing agents damage the IM and can cause spore killing without causing DPA release. Both the IM lipids and IM proteins can be targets for such spore killing.

The IM layer is also crucial for the process of spore germination. A number of proteins essential for spore germination, including the nutrient germinant receptors (GRs), the SpoVA proteins that facilitate DPA release during germination, and the GerD protein essential for GR-dependent germination, are generally considered to be present in or associated with the spore IM. Aspects of spore germination that are known and those that are still unknown have been extensively reviewed recently. The GRs, i.e., GerA, GerB, GerK, have been identified and quantified. These GRs have also been shown to respond to specific nutrient molecules. Along with proteins such as CwJ, SleB, YpeB, SpoVAD, the protease HtrC has been shown to play a pivotal role in spore germination. Yet, questions regarding, for instance, the in vivo interaction between different GRs, regulated efflux of cations, regulated DPA efflux from spores, and the coordination and regulation of cortex lytic enzyme activity remain unanswered. These unanswered questions spur the need for detailed proteomics studies of the IM to obtain an exhaustive analysis of the minimal protein composition of an IM-enriched fraction that is compatible with maintaining spore viability and identify unknown proteins putatively linking the above-mentioned key players of the spore germination process.

In the current study, we outline a robust protocol for spore IM isolation and coupled GeLC–MS/MS-based membrane proteomics. The most striking findings concerning the composition of the spore IM proteome are presented. The data obtained are discussed in the framework of open questions about spore germination and thus further the development of strategies for targeted inhibition of bacterial spore germination and subsequent outgrowth.

**MATERIALS AND METHODS**

**Culture and Sporulation Conditions**

*B. subtilis* strain 1A700 (trp2C) from a single colony was inoculated in tryptic soy broth (TSB), grown aerobically at 37 °C and 200 rpm until early exponential phase (OD600 0.3–0.4) was reached, and transferred to a defined minimal medium for sporulation buffered with 3-(N-morpholino)propanesulfonic acid (MOPS), as described previously. Cells were then diluted into fresh MOPS medium (20 mL) and grown until early exponential phase was reached, at which time they were transferred to larger volumes (250 mL) of MOPS medium. For vegetative cell preparation, cells were harvested upon reaching early exponential phase. For sporulation, cells were incubated for 96 h before the spores were harvested.

**Spore Inner Membrane Isolation**

Spores were harvested by centrifugation and washing with 0.1% Tween-80 and Milli-Q water. Purification of spores was done by centrifugation through a 20–50% HistoDenz gradient and washing with water. The purity of the spore crops was examined by phase-contrast microscopy.

**Electron Microscopy**

After each treatment, the spores or isolated membrane fraction were fixed in 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (McDowell fixative) and either directly negatively stained with uranyl acetate and imaged by TEM or Epon-embedded and sectioned. For sectioning, postfixation with 1% osmium tetroxide (OsO₄) Electron Microscopy Sciences, Hatfield, PA, USA) in cacodylate buffer was performed first. Subsequently, the spores were dehydrated in an alcohol series and embedded into Epon (LX-112 resin Ladd Research, Williston, VT, USA). Ultrathin Epon sections of 90 nm were collected on Formvar-coated grids, counterstained with uranyl acetate and lead citrate, and analyzed by transmission electron microscopy (TEM; FEI Tecnai T12).

**Sample Preparation for MS**

Membrane pellets from three replicate spores and three replicate vegetative cells were resuspended into 200–300 μL...
of water. Protein concentration was evaluated by the Micro BCA protein assay (Pierce). The yield of spore IM proteins was 50–100 μg from 100 to 150 mg dry weight of spores, which is comparable to previously reported IM isolations. Protein (50–100 μg) was loaded and separated by SDS-PAGE. SDS-PAGE was run on 10% Novex Tris-Glycine gels (THERMOScientific) at 125 V constant voltage with an XCell SureLock Mini-Cell (THERMOScientific). The gel was stained with Coomassie blue. The lanes containing membrane proteins were fractionated into nine pieces. Gel pieces went through reduction by 10 mM Dithiothreitol in 100 mM NH₄HCO₃ at 56°C for 1 h and alkylation by 55 mM iodoacetamide in 100 mM NH₄HCO₃ in the dark for 45 min. Proteins were digested by 12.5 μg/mL trypsin in 50 mM NH₄HCO₃ buffer overnight. Eluates of peptides were freeze-dried and finally reconstituted with 3% acetonitrile/0.1% trifluoroacetic acid for MS analysis.

**LC–MS/MS Analysis**

Mass spectrometry analysis of the peptide samples was performed with an amaZon Speed Iontrap with a CaptiveSpray ion source (Bruker) coupled with an EASY-nLC II (Proxeon, Thermo Scientific) chromatographic system. Peptides (100 ng) of each of the nine gel fractions from the three replicates of spore IM and from the three replicates of the vegetative cell isolates were injected and separated with an eluent flow of 300 nL/min on an EASY-Column 10 cm (SC 200 Thermo Scientific coupled with SC001 2 cm precolumn) using a 50 min gradient of 0–50% acetonitrile and 0.1% formic acid.

Some selected MS/MS instrument parameters were as follows: MS mass range, m/z 400–1500; MS/MS mass window, from m/z 100; precursor mass window selection, m/z 4.0; number of precursor ion selections, 5; CID in SmartFrag mode with variable energy; data-dependent acquisition with active exclusion after 1 spectrum with release after 30 s.

Raw MS/MS data of the gel peptide fractions were processed as multifile (MudPIT) with the MASCOT DISTILLER program, version 2.4.3.1 (64 bits), MDRO 2.4.3.0 (MATRIX science, London, UK). Peak picking for MS and MS/MS spectra was optimized for a mass resolution of up to 3500 (m/Δm) and 2500, respectively. Peaks were fitted to a simulated isotope distribution with a correlation threshold of 0.7 and with a minimum signal-to-noise ratio of 2. The processed data combined over the nine gel fractions were searched with the MASCOT server program 2.3.02 against B. subtilis strain 168 protein database from the UniProt consortium (January, 2014, release; 4244 entries in total) with the redundancy removed containing contaminants downloaded from the Mascot Web site collected by the Max Planck Institute of Biochemistry (Martinsried). The database was complemented with its corresponding decoy database for statistical analyses of peptide false discovery rate (FDR). Trypsin was used as the enzyme, and one missed cleavage was allowed. Carbamidomethylation of cysteine was used as a fixed modification, and oxidation of methionine, as a variable modification. Both peptide and MS/MS fragment mass tolerance was set to 0.3 Da. The MASCOT MudPIT peptide identification score was set to a cutoff of 18. At this cutoff, and based on the number of assigned decoy peptide sequences, a peptide false discovery rate (FDR) of ~2% for all analyses was obtained. The MASCOT protein identification reports were exported as XML and then imported in a custom-made VBA software program running in Microsoft Excel. The program facilitates organization and data mining of large sets of proteomics data.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD003268 and 10.6019/PXD003268.

Details of the MS analyses and the protein identifications are listed in the Supporting Information.

**Bioinformatics**

Membrane proteins were predicted using the following programs or databases: PSORTb 3.0 (http://www.psort.org/psortb/), Phobius (http://phobius.sbc.su.se/), BOMP (http://services.cbu.uib.no/tools/bomp), PRED-LIPO (http://bioinformatics.biol.uoa.gr/PRED-LIPO/), LocateP (http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py), and InterProScan 5 (http://www.ebi.ac.uk/interpro/). Identified proteins were categorized according to SubtiWiki (http://subtiwiki.uni-goettingen.de/).

**RESULTS AND DISCUSSION**

**Isolation of B. subtilis Spore Inner Membrane**

To investigate the B. subtilis spore IM proteome, we adapted procedures that have been used to identify the localization of several spore IM proteins to mass spectrometry-based proteomics approaches (Figure 1). Dormant spores made from a defined minimal medium were purified using density gradient centrifugation, and a spore purity of 99.5% was obtained.

Spores were first decoated using a harsh chemical treatment (see Materials and Methods). It was shown by Hudson et al. that decoating does not completely remove the coat and that the remnant will eventually be in the integument fraction. This is likely due to the high extent of cross-linking in coat proteins. Visualization of the spores by electron microscopy confirmed the presence of the coat after the first extraction (Figure 1). The contrast of the core is more intense using electron-dense staining (osmium and uranyl acetate), as this staining can better penetrate the spores after the extraction. It was also reported that the outer membrane is absent after the decoating treatment. This is probably ensured by the extensive washing steps, although in our hands DacA (penicillin-binding protein 5k), which was reported to localize to the OM, was identified in the sample obtained. Thus, we assume that by this procedure the extractable fraction of coat proteins was removed, leaving the insoluble portion with fissions that allow lysozyme to enter and degrade the cortex and germ cell wall. Electron micrographs of the lysozyme-treated spores show a dramatic expansion of the spore core, suggesting the degradation of the cortex and germ cell wall. Breakage of the decoated spores followed by differential centrifugation gave a final pellet enriched for the IM. Electron micrographs show that the final pellet consists of IM (Figures 1 and S3), whereas the integument pellet contains disrupted parts of the coat (Figure S4). To assess the efficiency of the lysozyme treatment, a muramic acid assay was deployed to probe for leftover peptidoglycan. The amount of muramic acid in the IM preparation was below the detection limit, whereas all muramic acid in the original spore was recovered in the supernatant collected following lysozyme treatment.
Identification of Candidate IM Proteins

Membrane proteins can be classified into different groups based on how they interact with the lipid bilayer: proteins with transmembrane spanning regions (integral membrane proteins), proteins covalently attached via fatty acids (lipoproteins) or glycosylphosphatidylinositol (GPI) anchors (in eukaryotes), and membrane-associated proteins that are bound non-covalently or transiently to the membrane. Protocols to isolate integral membrane and covalently linked membrane proteins usually aim at removing membrane-associated proteins and nonspecifically adhering cytoplasmic proteins by washing with high-salt and high-pH buffers. Following a standard membrane purification method, we were able to enrich our samples for spore IM integral membrane proteins and lipoproteins. It should be noted that it is virtually impossible to remove all cytoplasmic proteins, and there is the distinct possibility that some of these proteins spend part of their in vivo lifetime in the cell associated with the spore IM, hence resulting in their classification as membrane-associated proteins (vide infra); instead, the goal is to enrich integral membrane proteins, membrane-anchored proteins, and peripheral membrane proteins to boost the identification of low-abundance ones. Next, SDS-PAGE was used to prefractionate the proteins to reduce the complexity of the samples and thus enhance the sensitivity of identification. Tryptic peptides were generated by in-gel digestion and analyzed by LC-MS/MS.

As it is impractical to efficiently avoid co-isolation of cytoplasmic and core proteins in the membrane fraction, the identified proteins can be filtered further using bioinformatics tools that complement biochemical methods of subcellular localization. Results of six bioinformatic tools were included: Phobius is a combined transmembrane topology and signal peptide predictor. PRED-LIPO is a hidden Markov model method for the prediction of lipoprotein signal peptides of Gram-positive bacteria. PSORTb is a subcellular localization prediction program for bacterial and archaeal sequences using multiple analytical modules. LocateP combines many subcellular location predictors and distinguishes seven different subcellular locations within Gram-positive bacteria. BOMP is a program to predict β-barrel membrane proteins. Although β-barrel membrane proteins were thought to be located in the OM of Gram-negative bacteria, chloroplasts, and mitochondria, they were recently also reported in Gram-positive bacteria. InterPro provides functional analysis of proteins by classifying them into families and predicting domains and important sites. It combines protein signatures from a number of member databases. InterProScan, the software package that allows sequences to be scanned against InterPro’s signatures, was used. Each algorithm has its own trade-off between precision and recall. In order to reduce the omission rate, we assigned a protein as a membrane protein if any of the tools indicated that it is membrane-related. Out of the 929 proteins that were identified in at least two of the three replicates, 334 proteins were assigned as candidate membrane proteins (Table S3). Of these candidate membrane proteins, 46 have not been reported by previous proteomics studies in B. subtilis.

It is also worth noting that the uniqueness of the bacterial spore IM is not covered in the algorithms; hence, in addition to intrinsic errors in algorithm-based identifications, extra caution needs to be taken when considering their sensitivity and specificity.

The number of proteins identified is dramatically higher than that in the outer layers of the spore. With several reported studies, fewer proteins have been identified in the analyses of the whole spore proteome than in the present IM isolate. This demonstrates the efficiency of the present IM isolation, which overcomes dynamic range limitations of MS analyses of the whole spore proteome.

Vegetative Cell Membrane Proteome

There have been several studies on the proteome of B. subtilis vegetative cells. To validate that our methods are effective at identifying membrane proteins and to see whether under our culture conditions the vegetative cell proteome would turn out to be distinct from the spore IM, we performed B. subtilis vegetative cell membrane protein identification as well. Microscopic examination of the cell crops could not find spores or spore-forming cells. Classical microbiological techniques used on the vegetative cell-containing fraction identified fewer than 1 in 2000 cells as a spore. GeLC-MS/MS
analysis of three biological replicates of vegetative cell membrane revealed 1316 proteins (Table S2). Of these, 880 were identified in at least two replicates, 721 of which were among the previously reported *B. subtilis* vegetative membrane subproteomes, yet 42 of the remaining proteins were predicted to be membrane proteins (Table S4). These results illustrate that differences between growth conditions used in the various experiments reported in the literature result in differential vegetative cell membrane proteomes. The identifications in the sample enriched for the vegetative cell membrane were compared with the data obtained for the spore IM proteins. There was a large portion of proteins, general or membrane-predicted, that overlapped in both cell types as well as proteins that were unique to either sample set (Figure 2).

![Figure 2](image-url) Venn diagram of proteins identified from the *B. subtilis* spore IM isolation and vegetative cell membrane fraction. Membrane proteins were predicted by several bioinformatics tools (see Materials and Methods).

**Functional Categorization**

In order to obtain an overview of the functional distribution of identified proteins, protein identifications of the IM fraction as well as those of the vegetative cell membrane were categorized according to *SubtiWiki* (Figure 3; see Figure S1 for subcategories). Proteins identified were distributed throughout almost all of the groups. In many of the groups, spore IM proteins and vegetative cell membrane proteins represent an approximately similar number. The fact that about half of the proteins for each membrane are unique corroborates that the IM contains more proteins in the exponential and early post-exponential cell growth categories than the vegetative cell membrane. As expected, the IM contains more proteins in the categories of resistance against oxidative and electrophile stress; however, proteins in the cold stress proteins and coping with hyper-osmotic stress categories were more enriched for vegetative cells than spores, reflecting that the spores did not meet cold or osmotic stress during sporulation. Moreover, from an ecological point of view, it is also less likely that these protein classes will be encountered in spores because cold and osmotic stresses are less damaging to them. The number of DNA repair/recombination proteins in spores was twice that of vegetative cells. Interestingly, the membrane proteins category included proteins that are not predicted to be membrane proteins by us. These proteins are either peripheral membrane proteins (e.g., KtrC) or have been reported in other membrane proteome data but are, in fact, localized in the cytosol during most of their life cycle. In the following sections, we discuss a selection of proteins that has potentially significant roles in the physiology according to the categorization. Notice that the discussion in the following sections is not limited to identifications that were predicted to be membrane proteins.

**Expected IM Proteins**

A few proteins have been reported to be localized in the spore IM by immunoblotting (Table 1 and references cited therein). The GRs of *B. subtilis* are heterocomplexes formed by three (A, B, and C) subunits encoded by tricistronic operons. The A-subunit consists of five or six predicted membrane-spanning domains next to N- and C-terminal hydrophilic domains, the B-subunit comprises 10—12 transmembrane helices, and the C-subunit is a lipid-anchored protein. We identified GerBA, GerKA, GerKB, and GerKC with high confidence, and one of the replicates allowed us to identify GerAB and GerBC. Although Stewart et al. estimated that the number of GerAA and GerAC subunits per spore is higher than that of GerBA, GerBC, and GerKA subunits, these two proteins were not identified in our isolates. The proteins encoded by the spoVA operon are involved in DPA uptake during sporulation and DPA release during germination. We identified five of the seven proteins, including the hydrophilic SpoVAEb, in line with the western blot analysis results of Perez-Valdespino et al. That SpoVAB and SpoVAEb were missing could be due to their low molecular weight (15.2 and 12.3 kDa), as proteins of low molecular weight in the genome were less representative among the identifications (Figure S2). Western blot analysis has high sensitivity, but its drawback is obvious, as a specific antibody for each protein target is required.

We used MS to analyze the IM fraction from spores in a high-throughput manner and identified most of the reported proteins as well as those predicted to be present in the spore IM based on their primary amino acid sequence and reported functions. It is still possible to further optimize the methods to identify the missing ones, e.g., reducing complexity with more concentrated samples. In the following sections, we discuss a selection of proteins encoded by the *ger* operon involved in germinant binding, but the further downstream signal-transduction mechanisms that mediate germination have not been unraveled yet. The release of cations followed by DPA release and divalent cations early in spore germination suggests that one or more channels for these ions must be opened in the inner spore membrane upon binding of a germinant to its receptor. It is possible that germinant receptors activate certain antiporters in order to obtain a shift in the electrochemical gradient across the IM. We identified several transporter proteins involved in different cellular processes. More than 50% of the identified transporter proteins belonged to the ABC transporters family. Of these, proteins YugO (potassium transporter), MrpA (sodium/proton antiporter), CorA (magnesium transporter), ZnuA (Zinc uptake protein), which were reported to be upregulated during sporulation,
were identified only from the spore IM fraction and could play a major role in such efflux processes. In addition, BceB (bacitracin exporter), MdxG and MdxF (maltodextrin transporters permeases), RbsB (β-ribose binding uptake protein), CydC (ATP-binding/permease), GlpT (glycerol-3-phosphate transporter), YcnJ (copper transporter), YflS (malate transporter), and YmfD (bacillibactin transporter) were also identified from the spore IM fraction. It is noteworthy that bacitracin has been seen to affect the size of parasporal crystals and the spores of *Bacillus thuringiensis*, and bacillibactin siderophore has been obtained when *Bacillus anthracis* spores underwent germination and outgrowth in low-iron medium.

### Proteases

Proteases play important roles during both sporulation and germination. Protease inhibitors have been shown to inhibit spore germination. The membrane-anchored protein YpeB is essential for proper cortex-lytic enzyme SleB assembly into spores. SleB is located interior to the cortex in the dormant spore, and our results corroborate its close association with the IM. Membrane protein YpeB has been suggested to maintain SleB in an inactive state through YpeB’s PepSY domains. A recent in vivo and in vitro study showed that *B. anthracis* HtrC cleaves YpeB proteolytically to give a stable fragment. We detect HtrC in *B. subtilis* expressed by *yyxA* in our IM fraction. HtrC’s proteolytic action might disrupt interaction with YpeB and permit SleB’s cortex-lytic activity. A *B. subtilis htrC* mutant was also shown to have altered YpeB proteolysis; however, the results suggested that other proteases were also involved. Interestingly, in this context, we identified the uncharacterized membrane protein TseB (YpmB), which contains two PepSY domains and that might have an inhibitory function in cortex degradation. Further experimental evidence is needed to establish the function of inhibitory proteins and proteases in the activation of cortex-lytic proteins. Small, acid-soluble spore proteins (SASPs) are well-known to be degraded by the germination protease Gpr during germination. A more recent study showed that yet another protease, YmB (or TepA), conserved among spore-forming species, was dedicated to the degradation of a specialized family of SASPs. These germination proteases were co-puriﬁed in our spore IM fraction. Proteases that play regulatory functions during sporulation, such as SpoIVB and CtpB (for SigK), spoIIIAA (for SigG), and spoIIGA (for SigE) were not among the identifications, reflecting their spatially and temporally regulated expression. Functions of some proteases that we identiﬁed, such as CtpA and YmH, remain to be elucidated.

During germination, the spore coat, composed of proteins, needs to be breached and eventually shed. It has been suggested that degradation is caused by specific hydrolytic enzymes.
enzymes located within the spore integument. However, proteins responsible for this process have not been reported, and proteome analysis of the spore coat did not suggest a candidate. The protease YabG has been shown to be involved in coat protein modification but not in degradation. It is possible that some uncharacterized proteins or the unidentified proteins in the coat are the hydrolytic enzymes. Given that coat proteins are extensively cross-linked, unknown hydrolytic enzymes located within the spore integument are required for continuation of sporulation and are therefore conserved in the spore membrane.

**DNA Repair, Replication**

Spore DNA is well-protected against damage from UV, heat, and oxidizing agents. The α/β-type SASPs bind to negatively supercoiled spore DNA, reducing the chemical and enzymatic reactivities of the DNA. A certain number of DNA repair genes are expressed during sporulation and possibly exert their function upon spore germination. The involvement of nucleotide excision repair enzymes, the spore photoproduct lyase, and base excision repair proteins in the protection of spores against UV radiation and wet heat has been shown previously. In our analysis of the IM fraction, the nucleotide excision repair enzymes UvrA and UvrB, the base excision repair enzymes Nfo (YqfS) and Nth, the spore photoproduct lyase SpIB, and sporation specific UV-damage endonuclease YwjD were identified. In a similar manner, during outgrowth, the chromosomal DNA is required to relax rapidly to reactivate transcription. In addition to the degradation of the SASPs, increased helicase activity during the initial stages of outgrowth may be necessary. In this regard, we identified DNA 3′−5′ helicase HeD (YvgS), ATP-dependent helicase DinG, YpvA, and PcrA in our spore IM fraction. Concurrently, most of the above-mentioned proteins have been reported to be overexpressed during the initial stages of germination in a previous gene expression study. Additionally, before the onset of outgrowth, the DNA repair mechanisms scan the DNA for possible damage. Such damage needs to be repaired prior to outgrowth. The identified DNA repair proteins RecA, RecF, RecO, and RecN and the mismatch repair proteins MutS and MutSB may play a vital role in these processes.

**Other Resistance Proteins**

Besides the DNA recombination and replication machinery, many other proteins, enzymes, and transporters are also responsible for the survival of spores and cells. In our analysis, heat shock proteins such as CtsR, GroES, GroEL, and HtpG were identified in the spore IM fraction. Along with these and others, CssS, a two-component sensor kinase involved in the control of cellular responses to protein secretion stress, was also identified. Proteins like MihA, MihD, AzoR1, and others involved in resistance to oxidative and electrophilic stresses were also identified from the IM fraction of spores. Interestingly, proteins BshA, BshB, and BshC involved in bacillithiol synthesis were also observed. Bacillithiol synthesis was reported to be upregulated during spore outgrowth in B. anthracis. Speculatively, this may also be true in B. subtilis. In addition to protein LiaS, a two-component sensor kinase involved in response to bacitracin, protein BecB, a bacitracin ABC transporter (permease), was also identified in spores, and a high amount of presynthesized bacitracin is known to inhibit germination in Bacillus licheniformis. YkdK, a two-component sensor kinase, and putative multidrug resistance proteins YhcA and YitG were identified in spores, which may provide germinated spores with resistance against toxins. Additionally, SdpI, an immunity protein, was identified in both spore and vegetative fractions, being dominant in the spore fraction. This is a membrane-bound protein required to protect the sporulating cells from self-killing. B. subtilis cells, after nutrient limitation, delay the commitment to spore formation by killing the other nonsporulating cells and feeding on them. This killing is mediated by the exported toxic protein SdpC. We also identified SdpC in the spore IM fraction. Interestingly, SdpA and SdpB proteins, involved in the production and transport of SdpC, were identified only in our vegetative cell fraction. Thus, it is plausible that the SdpC and SdpI proteins are required for continuation of sporulation and are therefore conserved in the spore membrane.

**Others**

As reported previously in Bacillus megaterium spores, spermidine synthesis takes place during germination and outgrowth of spores. In our analysis of B. subtilis spores, we identified a couple of enzymes (SpeA and SpeE) that are part of the spermidine biosynthesis pathway (KEGG pathway analysis). In addition, an arginase (ArgI) was also identified, which could be involved in the conversion of arginine to ornithine. Ornithine can also be converted to putrescine (an intermediate in spermidine synthesis) by a predicted but not yet identified ornithine decarboxylase in B. subtilis. All of the above-mentioned enzymes have been identified in germinating B. subtilis spores in a recent work by Sinai et al. In our analysis of the B. subtilis spore IM fraction, enzymes involved in

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**Table 1. Proteins Reported or Predicted To Be Present on IM**

<table>
<thead>
<tr>
<th>protein</th>
<th>reported IM localized (refs)</th>
<th>identified in this study</th>
<th>function</th>
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<tr>
<td>GerBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GerBC</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GerKA</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GerKB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GerKC</td>
<td>55 b</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>SpoVAA</td>
<td>++</td>
<td></td>
<td>DPA uptake and release</td>
</tr>
<tr>
<td>SpoVAB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SpoVAC</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SpoVAD</td>
<td>22</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>SpoVAea</td>
<td>53</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>SpoVAeb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SpoVAF</td>
<td>53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GerD</td>
<td>23, 56</td>
<td>++</td>
<td>essential for GR assembly</td>
</tr>
<tr>
<td>SleB</td>
<td>57</td>
<td>++</td>
<td>cortex-lytic enzyme</td>
</tr>
<tr>
<td>YpeB</td>
<td>57</td>
<td>++</td>
<td>essential for SleB assembly</td>
</tr>
<tr>
<td>YhcN</td>
<td>58</td>
<td>++</td>
<td>unknown</td>
</tr>
<tr>
<td>PrkC</td>
<td>59</td>
<td>++</td>
<td>Ser/Thr membrane kinase and GR to peptidoglycan fragments</td>
</tr>
</tbody>
</table>

*Proteins in this table are either experimentally shown to be localized to the IM or are predicted as likely to be localized to the IM based on their function. These reports used B. subtilis except that GerAB was on Clostridium botulinum and GerKC was on Clostridium perfringens. +, identified in one replicate; ++, identified in at least two replicates.*

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DOI: 10.1021/acijpre4000976
J. Proteome Res. 2016, 15, 585–594
coenzyme A synthesis (according to KEGG pathway analysis), viz. PanB, PanC, PanE, CoaBC, CoaE, and IlvD, were identified. Incidentally, coenzyme A has been previously reported to be disulfide-linked to proteins in *B. megaterium* spores.76,77 Thus, CoA can play a role in the establishment of the structural integrity of spores and can also play an important role as a modulator of the metabolism that is operative in germinating and outgrowing spores, as has been suggested previously.78

**CONCLUSIONS**

Along with extensive studies of the biochemistry and function of individual proteins, the emergence of proteomics techniques, especially mass spectrometry-driven proteomics, has broadened our knowledge of spores in a more comprehensive way. Although extensive efforts have been devoted to the spore coat proteome and have led to a detailed understanding of spore structure and functions as well as wide applications, our present study is the first to report a spore IM proteome. The cell membrane harbors proteins that play key roles in various physiological processes, and the spore IM is not an exception. Because of the low abundance of IM proteins and the limited dynamic range of mass spectrometers, a sweeping query of the whole spore is unable to produce a satisfying number of IM proteins because many of them are masked by proteins of higher abundance from other layers. By fractionation and enrichment of the IM, we identified 336 membrane proteins along with 591 membrane-associated proteins, a number that is far beyond that of known spore proteins. Through a comparison with the vegetative cell membrane, the dynamics of the membrane proteome can also be seen. Functional categorization gives hints regarding proteins involved in different processes that need further study. Future spore IM proteomics studies will be focused on quantitation and topological determination of the IM proteins as well as interactions between different IM proteins, between proteins in the IM and other spore layers, and between IM proteins and small molecules such as germinants and inhibitory compounds.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00976.

Proteins identified in the *Bacillus subtilis* spore inner membrane fraction (Table S1) (XLSX)

Proteins identified in the *Bacillus subtilis* vegetative cell membrane fraction (Table S2) (XLSX)

Proteins identified in at least two replicates of the *Bacillus subtilis* spore inner membrane fraction and membrane protein predictions (Table S3); proteins identified in at least two replicates of the *Bacillus subtilis* vegetative cell membrane fraction and membrane protein predictions (Table S4) (XLSX)

Subcategorization of proteins according to SubtiWiki (Figure S1); mass distribution of identified proteins in three replicates of the spore inner membrane fraction (Figure S2) (XLSX)

Electron micrograph of the negatively stained *Bacillus subtilis* spore inner membrane fraction (Figure S3); electron microscopic analysis of spores during the procedure of the *Bacillus subtilis* spore inner membrane isolation (Figure S4) (PDF)

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**Author Contributions**

S.B. and C.G.d.K. contributed equally to this work. S.B., C.G.d.K., and L.J.d.K. participated in the design and coordination of the study and in supervising the drafting of the manuscript. L.Z. performed the experiments and wrote the manuscript. W.A. participated in drafting the manuscript. N.O. and H.v.V. performed a part of the experiments. H.L.D., N.v.d.W., and W.R. provided expert technical assistance.

**Funding**

L.Z. acknowledges the Erasmus Mundus program (EMEA3) and TNO (Healthy Living) for funding of his Ph.D. project.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank Chao Zhang and Xiyun Gan for their generous support on bioinformatics aspects, Hansuk Buncherd and Sacha Stelder for valuable advice, and Jolanda Verheul and Jos C. Arents for experimental assistance.

**ABBREVIATIONS**

MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; GeLC, gel electrophoresis liquid chromatography; MudPIT, multidimensional protein identification technique; IM, spore inner membrane; OM, spore outer membrane; DPA, dipicolinic acid; GR, germinant receptor

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