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Analysis of the Spore Membrane Proteome in *Clostridium perfringens* Implicates Cyanophycin in Spore Assembly

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ABSTRACT

Heat-resistant endospore formation plays an important role in *Clostridium perfringens*-associated foodborne illnesses. The spores allow the bacterium to survive heating during normal cooking processes, followed by germination and outgrowth of the bacterium in contaminated foods. To identify proteins associated with germination and other spore functions, a comparative spore membrane proteome analysis of dormant and germinated spores of *C. perfringens* strain SM101 was performed by using gel-based protein separation and liquid chromatography coupled with matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) mass spectrometry. A total of 494 proteins were identified, and 117 of them were predicted to be integral membrane or membrane-associated proteins. Among these membrane proteins, 16 and 26 were detected only in dormant and germinated spores, respectively. One protein that was detected only in germinated spore membranes was the enzyme cyanophycinase, a protease that cleaves the polymer cyanophycin, which is composed of L-arginine-poly(L-aspartic acid), to β-Asp-Arg. Genes encoding cyanophycinase and cyanophycin synthetase have been observed in many species of *Clostridium*, but their role has not been defined. To determine the function of cyanophycin in *C. perfringens*, a mutation was introduced into the *cphA* gene, encoding cyanophycin synthetase. In comparison to parent strain SM101, the spores of the mutant strain retained wild-type levels of heat resistance, but fewer spores were made, and they were smaller, suggesting that cyanophycin synthesis plays a role in spore assembly. Although cyanophycin could not be extracted from sporulating *C. perfringens* cells, an *Escherichia coli* strain expressing the *cphA* gene made copious amounts of cyanophycin, confirming that *cphA* encodes a cyanophycin synthetase.

IMPORTANCE

***Clostridium perfringens* is a common cause of food poisoning, and germination of spores after cooking is thought to play a significant role in the disease. How *C. perfringens* controls the germination process is still not completely understood. We characterized the proteome of the membranes from dormant and germinated spores and discovered that large-scale changes occur after germination is initiated. One of the proteins that was detected after germination was the enzyme cyanophycinase, which degrades the storage compound cyanophycin, which is found in cyanobacteria and other prokaryotes. A cyanophycin synthetase mutant was constructed and found to make spores with altered morphology but normal heat resistance, suggesting that cyanophycin plays a different role in *C. perfringens* than it does in cyanobacteria.**

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium that causes myonecrosis (gas gangrene) as well as acute food poisoning and nonfoodborne gastrointestinal diseases (1). Due to their general resistance properties (2), spores are believed to be the major means of transmission of this pathogen (3). After spore germination, *C. perfringens* has the capacity to quickly grow to high numbers in a variety of foodstuffs, with generation times as short as 8 min having been recorded (4). If ingested in high numbers, vegetative cells sporulate in the intestines and produce the *C. perfringens* enterotoxin (CPE), which is released upon lysis of the mother cell after sporulation is complete (5). While several studies have focused on the germination of *C. perfringens* spores (6–10), spore germination has been most intensively studied in *Bacillus* species. Based on the current model, the spore germination cascade is triggered by the interaction between the germinant and germinant receptors, and these germinant receptors are located in the spore inner membrane (11). It is believed that the interaction between germinants and germinant receptors changes the permeability of the spore inner membrane, which results in an efflux of ions (12). However, the signal transduction involved in this process is poorly understood. During ion flux, monovalent cations such as K^+, Na^+, and H^+ are released from the spore core to the environment, along with a large amount of calcium dipicolinic acid (Ca-DPA) (10, 12, 13). At least two ion channel proteins and transporters have been found to play a role in this stage, the spore inner membrane SpoVA proteins and the putative spore outer membrane Na^+/H^+-K^+ antiporters (10, 14). Later events in spore germination include the degradation of the spore cortex by germination-specific cortex-lytic enzymes and the degradation of core proteins by proteases, followed by full rehydration of the spore core and resumption of metabolism (15).

There are several known differences between the germination

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processes of *C. perfringens* and those of *Bacillus subtilis*. First, although both *C. perfringens* SM101 and *B. subtilis* spores germinate efficiently with polar uncharged amino acids (6), just potassium salts can trigger the germination of spores of strain SM101 (16). Second, *C. perfringens* SM101 contains only one classical germinant receptor complex, GerKABC, which contains three proteins that are homologues of the *B. subtilis* GerK proteins. However, the three GerK proteins in *C. perfringens* are not encoded by a tricistronic operon, as germinant receptors are in *B. subtilis*. Instead, *gerKB* is transcribed in the opposite direction of the bicistronic operon *gerKAC* (16). In addition, there is another GerA protein homologue, GerAA, encoded by a monocistronic locus in SM101. However, mutation of *gerAA* did not result in significant germination deficiency when tested with various known germinants (16).

To better understand the initiation of germination in *C. perfringens* and possibly identify proteins unique to *C. perfringens* spore germination, a mass spectrometry (MS) approach was used to analyze the spore membrane proteome in both dormant and germinated spores. Over 100 membrane proteins were identified, and their relative levels of detection were measured. These membrane proteins fell into several functional classes, including germination receptors and nutrient transport proteins. Surprisingly, the enzyme cyanophycinase (CphB) was detected only in germinated spore membranes. Cyanophycinase (17) breaks down the polymer cyanophycin, comprised of L-arginine-poly(L-aspartic acid), into β-Asp-Arg dipeptides. While cyanophycin has been most extensively studied as a storage polymer in cyanobacteria (18), it is also produced by many other bacteria, including species of *Clostridium* (19). The role of cyanophycin, beyond the logical assumption that it serves as a carbon and nitrogen reserve, has yet to be defined in the *Clostridia*. We therefore introduced a mutation into the *C. perfringens* SM101 gene encoding cyanophycin synthetase (*cphA*). Although the spores of the mutant strain retained wild-type (WT) levels of heat resistance, fewer spores were made, and the spores were smaller, implicating cyanophycin synthesis as a factor in spore assembly, a novel role for this compound in prokaryotes.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *C. perfringens* strain SM101 (20) is the sporulating strain used for this study. Three different media were used: brain heart infusion (BHI) medium (Difco), fluid thioglycolate (FTG) medium, and Duncan-Strong sporulation medium in the presence of 0.4% raffinose (DSSM) (21). *C. perfringens* was grown in a Coy anaerobic chamber at 37°C. *Escherichia coli* strain DH10B was used for all cloning experiments and was grown on Luria-Bertani phage medium.

Spore production. A frozen stock of *C. perfringens* SM101was streaked out on a freshly made BHI agar (1%) plate and incubated anaerobically at 37°C overnight. The next day, a single colony was picked; inoculated into liquid BHI medium; incubated until the culture entered exponential phase, ~8 h after initial inoculation; and then subcultured in liquid FTG medium (1:100 dilution). The culture grown overnight in FTG medium was subcultured in DSSM (1:200 dilution) and incubated for 7 days at 37°C.

Dormant spore purification. Three liters of sporulating culture was harvested by centrifugation at 5,000 × *g* for 10 min at 4°C, washed with cold deionized water 3 times, resuspended in cold deionized water, and kept on a shaker for 3 days at 4°C with a daily wash in cold deionized water. After the last wash, the spores were resuspended in 20% sodium diatrizoate (Sigma) at an optical density at 600 nm (OD_{600}) of 10. Aliquots of the spore suspension (4 ml) were layered on top of a 50% sodium diatrizoate solution in 50-ml polypropylene conical tubes (BD Falcon) and then centrifuged for 45 min at 6,871 × *g* at 25°C. Due to their higher density, only the dormant spores formed a pellet at the bottom of the tube, while germinated spores and cell debris remained above the 50% sodium diatrizoate layer. Dormant spores were harvested and washed with cold deionized water 5 times immediately after density gradient centrifugation. The OD_{600} of the spore suspension was measured again, and a portion (2 OD_{600} units) was then reserved for germination assays, with the remaining spores being aliquoted into two 1.7-ml centrifuge tubes. Liquid nitrogen was used to quick-freeze the spore pellet, which was then dried under a vacuum by using a refrigerated Vacufuge concentrator (Savant). One tube of spores was used as the dormant spore sample; the other one was used for preparing the germinated spore sample. A total of 3 independent batches of spores were prepared and assayed separately.

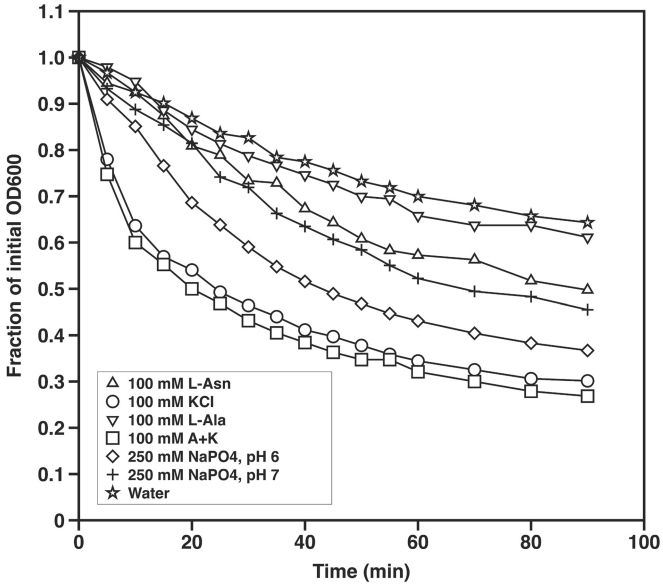

FIG 1 Response of *C. perfringens* spores to various germinants. Dormant spores were heat activated at 80°C for 10 min prior to germinant addition, and changes in the OD_{600} were monitored. A+K denotes L-asparagine and potassium.

Germination assay. Clean dormant spores were resuspended in cold deionized water at a final concentration of an OD_{600} of 1. The spores were heat activated at 75°C for 10 min and then quenched on ice for 5 min. Prewarmed water was mixed with the spore suspension (final OD_{600} of 0.2) and KCl (100 mM final concentration) to initiate the germination assay. As shown in Fig. 1, other germinants were used in assays to screen for the ideal germinant. Germination was carried out in a 37°C water bath with frequent gentle shaking to avoid spore sedimentation. The OD_{600} was measured every 2 min for 20 min. For the negative control, 100 μl of deionized water was used instead of 1 M KCl.

Germinated spore preparation. Dormant spores were heat activated and germinated with 100 mM KCl for 20 min as described previously (16). Germination was quenched by putting the spores on ice for 5 min, and the spores were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C and washed with cold deionized water 5 times. The germinated spore sample was freeze-dried as described above.

Spore membrane extraction. Dry spores were broken with 0.4-g glass beads (0.1-mm diameter) for 35 cycles at 4,600 rpm with 30 s of cooling on ice and 30 s of shaking (Crescent Wig-L-Bug MSD amalgamator). The disrupted spores were checked under a microscope to ensure that >80% of the spores were broken. The spore membrane was isolated by differential centrifugation (22). A small portion of the membrane preparation was subjected to amino acid analysis to determine the protein concentration

by using a previously described method (23), and the rest was freeze-dried as described above.

Gradient SDS-PAGE and gel slice preparation. The spore membrane protein was resuspended in loading buffer (Laemmli sample buffer [Bio-Rad] with 355 mM 2-mercaptoethanol) for a final protein concentration of 2 μg/μl. The sample was then heated in a boiling water bath for 10 min and cooled on ice for 10 min. Twenty-six micrograms of protein from each sample was loaded and separated by SDS-PAGE in a 10.5-to-14% gradient polyacrylamide gel (Criterion; Bio-Rad). The gel was then visualized by using Coomassie brilliant blue R-250 (Bio-Safe; Bio-Rad) and destained with deionized water. Each lane was hand cut into 12 slices, and the top slice was discarded. Some gel slices contained only one or two high-quantity protein bands, while other gel slices may have contained several low-quantity bands. This was to prevent high-quantity proteins from masking the detection of low-quantity proteins. Each gel piece was homogenized with a pellet pestle and destained (25 mM ammonium bicarbonate-acetonitrile [ACN] in a 1:1 ratio). Destained gel pieces were then dehydrated with ACN and dried in a vacuum centrifuge.

In-gel enzymatic digestion. Dry gel plugs were rehydrated in a trypsin solution (10 μg/ml trypsin and 25 mM ammonium bicarbonate) and digested at 37°C for 16 h. Peptides were then extracted by the addition of 500 μl of 0.1% trifluoroacetic acid (TFA) in 50% ACN and 15 min of sonication, dried under a vacuum, and resuspended in 40 μl 0.1% TFA in 2% ACN.

Separation and fractionation of tryptic peptides. Fifteen microliters of each peptide sample was injected into a sample loop that was in-line with an Eksigent NanoLC-2D high-performance liquid chromatography (LC) (HPLC) system and an Eksigent Ekspot matrix-assisted laser desorption ionization (MALDI) plate spotting robot. Peptides were eluted at a flow rate of 700 nl per min through a trap cartridge (Magic C_{18} AQ, 200 Å, 3 μm; Bruker) and a self-packed IntegraFrit 50- by 0.1-mm column (New Objective). The mobile phase (solvent A) was 2% ACN–98% water supplemented with 0.1% TFA, and solvent B was 98% ACN–2% water supplemented with 0.1% TFA in the following gradient mode: first 10 min, 0 to 10% solvent B; min 10 to 100, 10% to 36% solvent B (linear increase over time); min 100 to 101, 36% to 75% solvent B; and min 101 to 150, 75% solvent B. The column eluate from between 53 and 149 min was spotted onto 384 spots at 15 s per spot.

MALDI-TOF/TOF MS analysis. A freshly made matrix solution (4 mg of ACN-washed alpha-cyano-4-hydroxycinnamic acid, 50% ACN, 10 mM NH_4Cl, 0.1% TFA) was spotted onto the plate and air dried right before analysis by MALDI-tandem time of flight (MALDI-TOF/TOF) MS (4800 MALDI-TOF/TOF instrument; AB Sciex). The instrument was operated at 1 kV in the positive-ion reflector mode and calibrated with an internal calibrant. MS spectra were acquired across the *m/z* range of 800 to 4,000 from 1,000 laser shots. The 15 most abundant precursor ions with a minimum signal-to-noise ratio of >50 were selected for subsequent tandem MS (MS/MS) analyses. The MS/MS spectra, each acquired by using 3,000 laser shots, were further processed.

Data analysis and protein identification. MS/MS spectra were interrogated by using ProteinPilot 4.0 (AB Sciex) for searching against the *C. perfringens* UniProt database (downloaded October 2013) appended with the reverse decoy and contaminants databases. The following parameters were used for ProteinPilot analysis: the unused score must be no less than 1, and the number of detected peptides of high quality (with at least 95% confidence) must be no less than 1 (which corresponded to a protein identification confidence of 90%), with the false discovery rate for protein identification set at 5%. Finally, the protein must be identified at least twice in a total of three independent replicates.

Analysis of relative levels of detectability of spore membrane proteins. We used the MASCOT online search engine (Matrix Science) to match tandem mass spectra with peptide sequences from the *C. perfringens* UniProt database. The triplicates from each sample group were pooled, and the resulting data were further evaluated with Scaffold 3.0 software (Proteome Software), using spectral counting for quantification. The relative levels of detectability of those membrane proteins (predicted by PSPRTb 3.0) that were seen in both dormant and germinated spore samples using ProteinPilot software were analyzed. Only those proteins that changed at least 2-fold in their levels of detectability during spore germination were considered significantly changed.

Construction and complementation of a *cphA* mutant. A 1,110-bp fragment internal to the *cphA* gene was amplified by using primers OHL161 (5′-GTTATTGTCGACGAGAGCATAGGGCCATCAACAAAGGC-3′) and OHL62 (5′-CTTATTGAATTCTTTAACTCCAGAAGTCGTTGTACAC), which contain SalI and EcoRI sites, respectively. The PCR product was digested with SalI and EcoRI and ligated to SalI-EcoRI-digested plasmid pSM300, a suicide plasmid carrying an *ermB* resistance gene (24), to produce pHLL68. Fifty micrograms of purified pHLL68 was used to transform strain SM101 to erythromycin resistance. The insertion into the *cphA* gene was confirmed by using primers OHL153 (5′-CCGCGGCTTTTGAGGAGGAAAGAAATAAAAATGAAG-3′), which anneals upstream of the region used for mutagenesis, and OJV59 (5′-TATGCTTCGAGCTCCTATGTTGTGTGGAATTGTGAG-3′), which anneals in the *ermB* gene of pSM300. A PCR product of the predicted size was produced from chromosomal DNA of *cphA* mutant strain HLL68. Complementation of the insertion mutation in *cphA* was accomplished by PCR amplification of the entire *cphBA* operon and its putative native promoter using primers OHL163 (5′-AGCAGTGGTACCAAAAAAAGAGTTTTACGATATTATGTC-3′) and OHL164 (5′-AAATTAGTCGACCTATATGCTAGCACTTTTTAAATC-3′), which contain KpnI and SalI sites, respectively. This included the region 112 bp 5 prime to the initiation codon of *cphB* and encompassed the last 13 bp of the flanking gene *CPR_2189*. The PCR product and the *E. coli-C. perfringens* shuttle vector pJIR750 (25) were digested with SalI and EcoRI and ligated to produce pHLL69. pHLL69, which carries the *catP* gene, was placed into strain HLL68 by electroporation and selection on plates containing erythromycin and chloramphenicol.

Construction of a *cphB-gusA* transcriptional fusion vector. The promoter region of the *cphBA* operon was amplified by PCR using primers OHL167 (5′-GATTCTCTCGAGGTTGGCGATAAAATTATGATTCCAGG-3′) and OHL168 (5′-CACCCTCTGCAGCTTCTGCTCCACCTATTATTATTAA-3′), which contain XhoI and PstI sites, respectively. This PCR product contained 141 bp upstream of the *cphB* gene, including 42 bp at the end of *CPR_2189*, and the first 23 codons of the *cphB* gene. The PCR product was ligated to the PCR cloning vector pGEM-T Easy (Promega) to form pHLL70, which was then digested with XhoI and PstI, followed by ligation to XhoI-PstI-digested pSM240 to form pHLL71. pSM240 is an *E. coli-C. perfringens* shuttle vector that contains a promoterless *gusA* gene from *E. coli*, which encodes the enzyme β-glucuronidase (26). β-Glucuronidase assays were carried out as previously described (21).

Expression of the *cphA* gene in *E. coli* and detection of cyanophycin. Primers OSM272 (5′-GGTGAATTCTGAGGAGGAAAGAAATAAAAATG-3′) and OHL164, which contain EcoRI and SalI sites, respectively, were used to amplify the *cphA* gene from strain SM101 chromosomal DNA by PCR. The PCR product was ligated to the PCR cloning vector pCR-Blunt II-TOPO (Invitrogen) to create pSM327, which was then digested with EcoRI and SalI, and the *cphA* DNA was ligated to EcoRI- and SalI-digested pBAD30 to create pSM328. pBAD30 contains an arabinose-inducible promoter, which can be used to generate high levels of transcription in *E. coli* cells (27). pSM328 was transformed into *E. coli* strain DH10B by electroporation. Two independent 100-ml cultures of DH10B(pSM328), grown with shaking at 37°C to an OD_{600} of 0.4, were induced by the addition of arabinose to a final concentration of 10 mM. The cultures were allowed to grow for four more hours and then harvested by centrifugation and frozen at −20°C. The cell pellets were thawed, suspended in Dulbecco's phosphate-buffered saline (DPBS), and lysed by sonication. Soluble and insoluble cyanophycin were purified from the whole-cell extracts as previously described (28). The amino acid content of purified cyanophycin was determined by acid hydrolysis and amino

acid analysis as previously described (23), with comparison to mixed and individual amino acid standards (Sigma).

Germination efficiency and heat resistance. For germination efficiency and heat resistance assays, spores from strains SM101, HLL68 (*cphA*), and HLL68(pHLL69) were prepared in triplicate. Cultures were grown overnight in FTG medium and then inoculated into DSSM at a 1:200 dilution and grown overnight at 37°C. Spores were prepared by multiple washes in sterile distilled water until they were >98% free of nonsporulating cells and cell debris, as determined by light microscopy. The germination efficiency with 100 mM KCl as the germinant was measured as follows. One-half of the spore suspension was washed 2 times in cold distilled water and resuspend in 0.45 ml of distilled water. Spores were activated by heating in microcentrifuge tubes for 10 min in a 75°C water bath and then cooled on ice for 10 min. Fifty microliters of 1 M KCl was added to half of the tubes (plus germinant), and 50 μl of water was added to the others (minus germinant). The tubes were incubated at 37°C for 20 min, cooled on ice for 10 min, and then heated in a 75°C water bath for 10 min before serial dilution and plating onto BHI medium with agar. The number of CFU per milliliter in the tubes lacking a germinant was divided by the number of CFU per milliliter in the tubes with a germinant and multiplied by 100 to calculate the germination efficiency. Heat resistance was measured by heating some spore suspensions at 75°C and others at 85°C for 10 min, followed by cooling on ice for 10 min, serial dilution, and plating onto BHI medium with agar. The number of heat-resistant spores per milliliter of culture for three independent samples was then calculated for comparison between the mutant and strain SM101.

Light and electron microscopy. Phase-contrast images were made on an Olympus IX71 microscope using the Applied Precision SoftWorx image capture program. Total spore and inner core lengths were measured along the long axis of phase-bright spores by using the two-point measurement application in the GNU Image Manipulation Program (GIMP) (v. 2.8.10) software package.

For transmission electron microscopy, purified spores were fixed in 2% glutaraldehyde, embedded, sectioned, and stained as previously described (29, 30). Samples were imaged on a JEOL JEM-1400 transmission electron microscope.

Statistics. Statistical analyses (Student's two-tailed *t* tests) were done by using InStat 3 or Prism 6.0 software (GraphPad, Inc.) For all statistical analyses, *P* values of <0.05 were considered significant.

RESULTS

Response of *C. perfringens* spores to various germinants. Several germinants of *C. perfringens* spores have been reported (16), with potassium (K^+) being considered a very efficient germinant for *C. perfringens* SM101 spores (16). Assays with different germinants confirmed that 100 mM KCl efficiently triggered the germination of >95% of the spores within 20 min, with a combination of potassium and L-asparagine producing a slightly higher efficiency (Fig. 1). Sodium phosphate buffer can also trigger germination (Fig. 1). For germinated spore membrane preparations, 100 mM KCl was used as the sole germinant.

Differential spore membrane proteomics analysis using MALDI-TOF MS/MS. Spore membrane proteins were separated by using SDS-PAGE gradient gels. Each lane was cut into 12 pieces for protein identification (Fig. 2). The gel slices containing membrane proteins from both dormant and germinated spores were ground and treated with trypsin. Peptides were extracted and analyzed by MALDI-TOF MS/MS. This search strategy resulted in the identification of 479 proteins. There were 393 and 402 proteins from dormant spore samples (see Table S1 in the supplemental material) and germinated spore samples (see Table S2 in the supplemental material), respectively, with 316 proteins being detected in both spore types. In this report, membrane protein is used as a general term to include all lipoproteins, membrane-associated proteins, and integral membrane proteins (IMPs). A total of 117 predicted membrane proteins were found by using PSORTb 3.0 software (31) (see Table S3 in the supplemental material). While 75 of these proteins were detected in both samples, 16 were detected only in dormant spore samples, and 26 were detected only in germinated spore samples (see Table S4 in the supplemental material). The program PSORTb 3.0 was used to identify the number of transmembrane helices (TMHs) in these 117 membrane proteins (Fig. 3). Among the 26 proteins containing no TMH, 25 were predicted to be lipoproteins by both PRED-LIPO and LipoP 1.0 (32, 33). The MurG protein (CPR_2034) does not contain any TMH, but its homologue in *E. coli* has been shown to be a membrane-associated protein (34). Of 22 proteins containing just one TMH, 6 were predicted to be lipoproteins. The remaining 69 proteins that contain more than 1 TMH were considered either IMPs or membrane-associated proteins.

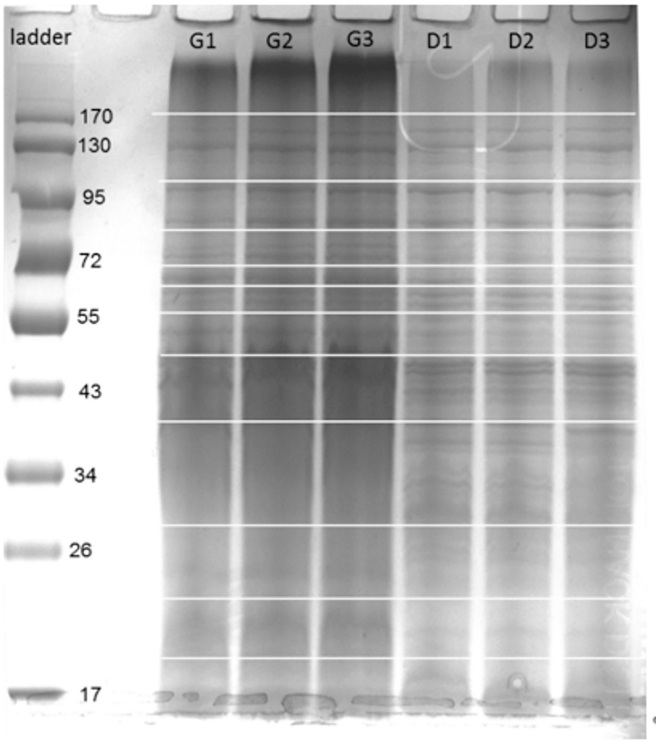

FIG 2 SDS-PAGE analysis of *C. perfringens* spore membrane protein preparations. Triplicate samples of dormant and germinated spore membrane proteins were separated by SDS-PAGE, and 11 gel slices, indicated by lines, from each sample were excised for proteome analysis. G, germinated spore membrane protein; D, dormant spore membrane protein.

Analysis of relative levels of detectability of spore membrane proteins. The determination of relative protein levels of detectability was performed by using the MASCOT search engine and the Scaffold proteomic data-processing package. Among the 75 membrane proteins that were identified in both dormant and germinated spore samples by ProteinPilot, only 71 were detected in both dormant and germinated spore samples by using MASCOT software. The other 4 proteins were detected only in germinated spore samples by using MASCOT software, and we consider them to have decreased levels of detectability during germination. Among the 71 membrane proteins found in both dormant and

FIG 3 Venn diagram of membrane protein identification. The diagram shows the TMH distribution of all membrane proteins identified from both dormant and germinated spore samples, according to PSORTb 3.0.

germinated samples by MASCOT, 24 of them showed relative levels of detectability that were changed by at least 2-fold during germination (see Table S4 in the supplemental material).

Cyanophycinase/cyanophycin synthetase. The enzyme cyanophycinase was detected in germinating spore membranes but not in those of dormant spores (see Table S4 in the supplemental material). Cyanophycinase is a specific protease that breaks down the nonribosomally synthesized polymer cyanophycin (often called CGP for cyanophycin granule polypeptide), which is composed of L-arginine-poly(L-aspartic acid), to β-Asp-Arg. Examination of the genome sequence of strain SM101 revealed the presence of a putative operon composed of the *cphB-cphA* genes, encoding cyanophycinase and cyanophycin synthetase, respectively (Fig. 4A), which were previously reported (19). To determine if cyanophycinase synthesis is linked to the sporulation cycle, a fusion of the *cphB* promoter region to the reporter gene *gusA* from *E. coli*, which encodes a β-glucuronidase (35), was constructed. In batch culture, the fusion was expressed in cells growing in DSSM at 5 h and 8 h postinoculation but not in the nonsporulating medium PGY (proteose peptone, glucose, yeast extract) at 8 h (see Fig. S1 in the supplemental material), suggesting a link to sporulation. To confirm this, cells were cultured in DSSM, and samples were taken every 30 or 60 min for 9 h. This fusion showed expression above background levels at between 3 and 4 h postinoculation, which corresponded to the end of logarithmic growth and the onset of stationary phase (Fig. 4B). Based on the use of toluene to permeabilize the membrane (21), we hypothesize that β-glucuronidase activity could be detected from the vegetative cells and the developing spore up to about stage III of sporulation.

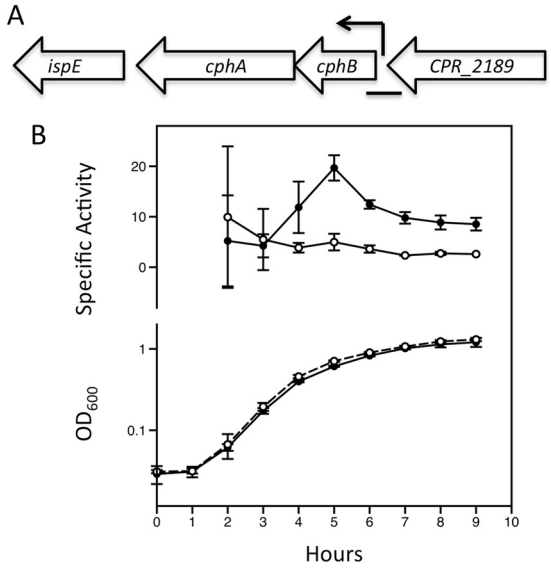

FIG 4 (A) Gene arrangement in the *cphBA* operon in strain SM101. The thin arrow points to the location of a putative promoter for the *cphBA* operon, and the bar represents the region tested for promoter activity in panel B. (B) Expression of the *cphBA* promoter (measured as specific activity of β-glucuronidase) (top) and growth (bottom) of strain SM101 with pHLL71 (*cphB-gusA*) (closed circles) and pSM240 (vector control) (open circles).

The presence of cyanophycinase in the germinating spore membranes suggested that cyanophycin may be present in sporulating cells, but repeated attempts to isolate the insoluble and soluble (28) forms of cyanophycin from sporulating cell extracts and spent sporulation medium were unsuccessful. To confirm that the *cphA* gene encodes an actual cyanophycin synthetase, we cloned the *cphA* gene into a vector, pBAD30, that allows high levels of expression in *E. coli*. After induction, significant amounts of cyanophycin that was soluble at neutral pH (referred to as "soluble") but not insoluble cyanophycin were purified from cell extracts. The composition of the purified soluble cyanophycin was 49.5 mol% ± 0.7 mol% aspartate, 46.5 mol% ± 0.7 mol% arginine, and 3.5 mol% ± 0.7 mol% lysine.

A plasmid insertion mutation was created in the *cphA* gene of strain SM101 (see Materials and Methods). Cyanophycin often appears in cyanobacteria as insoluble granules visible by light and electron microscopy. Transmission electron micrograph images of sporulating WT and *cphA* mutant cells showed the presence of multiple intracellular granules, but there was no obvious difference in the number or form of the granules seen with the two strains (data not shown).

The sporulation efficiency of triplicate samples, gauged by the number of heat-resistant spores produced per milliliter after culture overnight, was ~3 times higher for strain SM101 than for the *cphA* mutant strain ($1.2 \times 10^6 \pm 6.6 \times 10^5$ versus $4.6 \times 10^5 \pm 2.1 \times 10^5$; $P = 0.0317$). Purified spores from parental strain SM101 and the *cphA* mutant strain were tested for differences in heat resistance at 75°C for 10 min by first counting the number of total spores in a hemocytometer and comparing this to the number that survived heat treatment, but no significant difference was observed (23% for SM101 and 17% for the *cphA* mutant). Similar results were obtained after incubation at 85°C for 10 min (data not

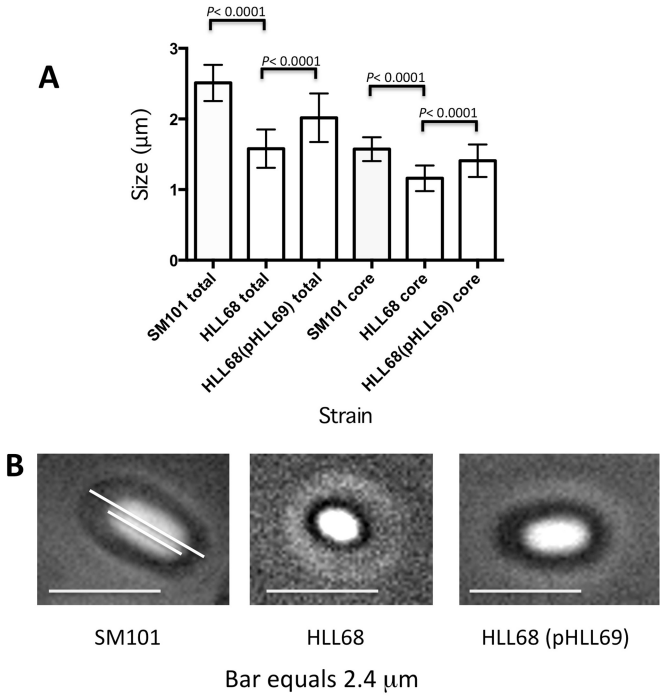

FIG 5 (A) Spore sizes measured by phase-contrast light microscopy of dormant spores. (B) Representative images of dormant spores of the three strains shown in panel A. The longer slanted line in the left panel indicates the total length of the spore, and the shorter slanted line indicates the phase-bright core spore. The light ring around the HLL68 spore is a phase-contrast artifact.

shown). Also, no significant differences were observed in spore germination efficiency between the two strains (both >99%).

Measurements of purified spores showed there was a significant decrease in the average length of the long axis of the spores made by the *cphA* mutant in comparison to those of strain SM101, with the complemented strain having intermediate-length spores (Fig. 5A). The same was true for the phase-bright core of purified spores (Fig. 5A). One consistent difference visible in phase-contrast images was the increased size of the space between the phase-bright core and the spore coat in the WT strain in comparison to the *cphA* mutant (Fig. 5B). To determine if there were ultrastructural differences in spores made by the wild-type strain versus the *cphA* mutant strain, thin sections of purified spores were imaged on an electron microscope at high magnification. There was a noticeable difference in the average sizes of the spores, with strain SM101 spores larger than HLL68(pHLL69) spores, which were themselves larger than those made by strain HLL68 (Fig. 6). Spores of strain HLL68 appear to be more spherical than those of strain SM101, which may account for the size differences measured by light microscopy. However, we could not detect any obvious differences in the components (i.e., core, cortex, and spore coats) of individual spores (Fig. 6).

Since differences in spore size could result in differences of the abilities of spores to survive over longer time periods, we measured the viability of spores in sporulation medium over 5 days but did not observe a statistically significant difference between the *cphA* mutant and WT strains (see Fig. S2 in the supplemental material).

One possible role for cyanophycin in spore function could be that it serves as a nitrogen storage source during the outgrowth of spores in nutrient-poor medium. To test this, we germinated purified spores by heating them at 75°C for 10 min in distilled anaerobic water, followed immediately by the addition of an equal volume of anaerobic DPBS to stimulate germination in the absence of carbon and nitrogen sources. A portion of the germinated spores was then plated on medium to determine the number of CFU, and the remaining spores were incubated for 16 h at 37°C in an anaerobic chamber. After this time, the germinated spores were plated, and the number of CFU was determined, but there was no significant difference between strain SM101 and the *cphA* mutant (2.5% ± 2.4% survival for SM101 versus 9.2% ± 4.1% survival for the *cphA* mutant; $P = 0.2485$). However, measurement of the sizes of the germinated spores that were incubated for 16 h indicated that the sizes of the germinated spores of WT strain SM101 were equal to those of the dormant spores, but in the *cphA* mutant strain, the germinated spores were smaller than the dormant spores (see Fig. S3 in the supplemental material). In summary, although the *cphA* mutant produces smaller spores than the WT strain, the resistance properties of these spores appear to be similar for the environmental and nutritional stresses tested.

DISCUSSION

The availability of whole-genome sequences, especially those of pathogenic bacteria, has given us the ability to perform proteomic studies on these organisms. Extensive proteome studies have been done in *Bacillus* to identify total spore proteins, coat proteins, or germination-specific proteins (36–39). Those studies established the basic map of spore proteins, with the majority of them being cytoplasmic proteins. There are also several comprehensive transcriptional studies of temporal gene expression during spore germination, for both *Bacillus* and *Clostridium* species (40, 41). However, there are still gaps with respect to the molecular mechanism of spore germination, especially in the early stages, and the spore inner membrane proteins are associated with these early events (42). In this study, we characterized the spore membrane proteins in the enterotoxin-producing food poisoning bacterium *C. perfringens* SM101 in both dormant and germinated spores.

To provide further insight into those spore membrane proteins that changed at least 2-fold in levels of detectability from dormant to germinated spores, their functional categories were examined by using the Clusters of Orthologous Groups (COG) protein database and data reported previously (43). These 70 membrane proteins fell into 9 major functional groups (Table 1). Details about functional categorization are shown in Table S3 in the supplemental material. This is the first comprehensive proteomic study specifically focusing on the spore membrane in a *Clostridium* species. By coupling SDS-PAGE and LC-MALDI-TOF MS/MS, a list of the spore membrane proteins was developed, and a comparison of their relative levels of detectability before and after germination helped reveal the proteins that may be actively involved in spore germination processes.

Of the 70 proteins that had 2-fold differences in detectability between dormant and germinated spores, 46 were at increased levels and 24 were at decreased levels. The first questions that arise are, where did the 46 proteins come from, and were they synthesized *de novo* in the 20 min after germination was initiated or were they present in dormant spores in some form that was not detectable by using our methods? In a recent study examining the time line of events in germinating spores of *B. subtilis*, translation of

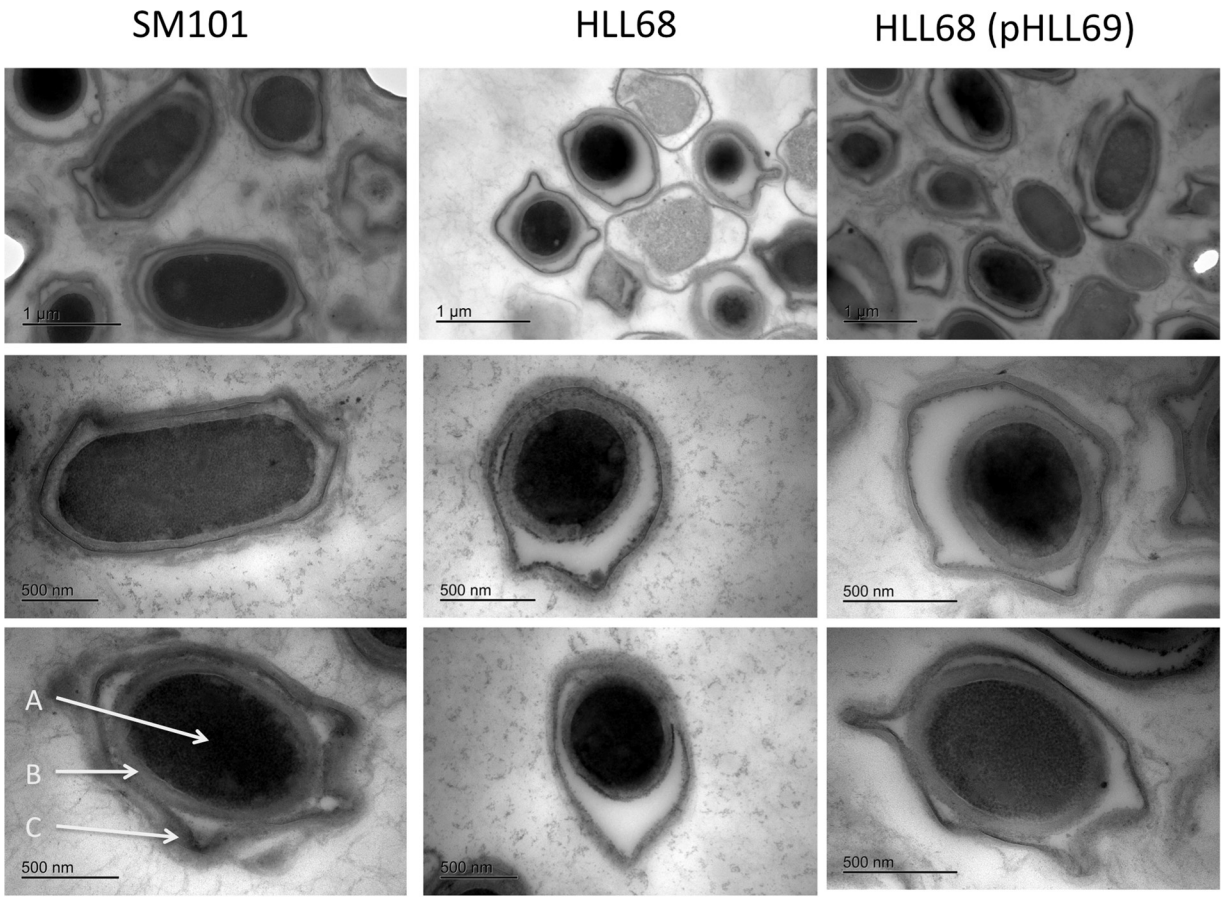

FIG 6 Transmission electron microscopy of spore cross sections. Representative images from each of the indicated strains are shown. (A) Spore core; (B) cortex layer; (C) coat layers.

TABLE 1 Summary of functions of differentially detected membrane proteins

Functional group[a]	No. of proteins showing increased detection[b]	No. of proteins showing decreased detection[c]
ABC transporter, general transport system	23	9
Protein secretion, translocation, degradation, chaperones	7	1
Cell division, cell wall and membrane biogenesis	6	1
Energy production and conversion	2	2
DNA replication, repair, transcription, translation	0	3
Signal transduction mechanism	0	1
Germination receptor	0	2
Phage protein	1	0
Unknown function	7	5
Total	46	24

[a] Function categorization analysis is based on data in the COG database and data reported in the literature.
[b] Proteins showing increased detection by at least 2-fold in germinated versus dormant spore samples.
[c] Proteins showing decreased detection by at least 2-fold in germinated versus dormant spore samples.

some proteins was detected in the first 30 min and one was detected as soon as 5 min after the addition of a germinant to total spore protein extracts (i.e., not just the membrane) (44). However, given the large number of proteins and their location, i.e., associated with the membrane, it seems more likely that in *C. perfringens*, they were present in dormant spores in some form that was not detectable by using our methods. Two scenarios seem plausible to explain the increased levels of protein: (i) the proteins could not be efficiently extracted from the dormant spore membranes due to some modification of their physical state that is relieved by the germination process, or (ii) they were not actually associated with the membranes in dormant spores.

C. perfringens contains four known germinant receptor protein homologues, GerAA, GerKA, GerKB, and GerKC. GerKC is considered to be a lipoprotein associated with the spore inner membrane (45, 46), whereas GerAA, GerKA, and GerKB are integral membrane proteins containing several transmembrane segments (16). All four germinant receptor proteins were detected, with only GerKB exhibiting a significant change in levels, with an almost 10-fold decrease in germinated spores. It is widely accepted that the germination commitment is irreversible once germinant receptors receive the signal from their germinants, and this commitment cannot be aborted by removing the germinants or by blocking the germinant receptors (42). It appears unlikely that these germinant receptors were still stored in the spore inner

membrane since they are not required for vegetative growth and were expressed only under the control of the sporulation sigma factor SigG (47–49). We hypothesize that the GerKB germinant receptors were degraded as part of the commitment to germination, possibly by the AAA protease FtsH, which showed higher levels of detectability in germinated spores. In *B. subtilis*, detection of the "C" subunits of the Ger receptors (GerAC, GerBC, and GerKC) decreased ~2-fold during germination (22). It is not known if the reduced detection is due to degradation or reduced association with the membrane during purification.

It was reported previously that mutation of a putative Na^+/H^+-K^+ antiporter, GerO (CPR_0227), containing 10 potential membrane-spanning domains, negatively affected spore germination and outgrowth (10). However, translation of GerO was observed only in the mother cell compartment and not in the forespore (10). In our proteomic study, this protein was not detected in dormant or germinated spore samples. This result supports the hypothesis that GerO is not located in the spore membrane or perhaps is not located in the spore at all.

The largest portion of the membrane proteins whose levels changed significantly during spore germination are proteins involved in transport processes (see Table S3 in the supplemental material). This includes classical ABC transporter complexes, ion transporters, permease proteins, the phosphotransferase system (PTS), transporters for other metabolites, and putative transporters. It is not surprising to see increased levels of several ion transporters in germinated spore samples considering the observation of a large efflux of cations during the early stages of germination in *Bacillus* species (12). Mutagenesis studies of several ion transporters have also shown that ion transport plays an important role in spore germination (10, 50). It is possible that several of the identified transporters, such as the cation efflux superfamily protein CPR_0983, the cation-transporting ATPase CPR_2026, and the ion compound ABC transporter CPR_1115, serve as channels or gates for cation efflux during spore germination, especially the protein encoded by *CPR_2026*, which shares 47% identity with the calcium pump ATPase YloB from *B. subtilis*. It was reported previously that deletion of YloB resulted in a partial loss of spore resistance and also a lower germination rate (51). This might be due to the inefficient uptake of calcium at the sporulation stage to form high concentrations of Ca-DPA in the spore core and the inefficient release of Ca^{2+} at the germination step. The CPR_2026 protein is similar to YloB in that it contains 9 out of the 10 conserved residues in the eukaryotic Ca^{2+} transporters (51), which are involved in the coordination of the two calcium ions that bind in the transmembrane domain.

There were relatively few membrane proteins that showed lower levels of detectability in germinated spores than in dormant spores (see Table S4 in the supplemental material). This list includes two ATP synthase subunits, AtpF and AtpB; elongation factor 4 (LepA); and the transcription termination factor Rho. It was found previously that in *Bacillus thuringiensis*, the translation of most ATP synthase subunits was upregulated during late sporulation to meet energy demands (52). We believe that the ATP synthase subunits were present at high levels during sporulation after being carried over from mother cells to the spores.

The enzyme cyanophycinase was detected in the membranes of germinating spores but not dormant spores. A *cphB-gusA* fusion was expressed during the end of log phase/beginning of stationary phase in *C. perfringens* strain SM101 (Fig. 4B). The expression of the *cphBA* operon during sporulation in strain SM101 was independently observed in a genome-wide transcriptional profile of this strain during the sporulation cycle (53). To our knowledge, these are the first reports linking cyanophycin synthesis with endospore formation. While cyanophycinase is not predicted to be an integral membrane protein, it is predicted to be membrane associated (31), likely due to a high level of predicted amphihelicity (54), which may explain why it was detected in purified spore membranes.

Since cyanophycinase is a highly specific protease that acts only on the polymer cyanophycin, it is suggested that cyanophycin itself may play a role in *C. perfringens* spore functions. Cyanophycin is a carbon and nitrogen storage compound found in diverse bacterial species but has been studied extensively mainly in cyanobacteria. The third enzyme in cyanophycinase metabolism is a specific peptidase that cleaves the β-Asp-Arg dipeptide into free Asp and Arg (19). We have detected a homologue of this enzyme, an isoaspartyl dipeptidase, in the genome of *C. perfringens* strain SM101 at the *CPR_2351* locus, suggesting that *C. perfringens* can synthesize and completely break down cyanophycin.

We could not purify cyanophycin from sporulating cells of strain SM101. However, expression of the *cphA* gene in *E. coli* led to the production of significant quantities of a cyanophycin that was soluble at neutral pH and was composed of 49.5 mol% ± 0.7 mol% aspartate, 46.5 mol% ± 0.7 mol% arginine, and 3.5 mol% ± 0.7 mol% lysine. These ratios suggest that this is a typical cyanophycin polymer with an aspartate backbone and arginine and lysine side chains, since aspartate accounts for 50% of the residues and the other two amino acids account for the remaining 50%. The solubility of cyanophycin is affected by the lysine content (28), and recombinant *cphA* genes from other bacteria expressed in *E. coli* produce soluble cyanophycin with increased lysine content (55). This makes it difficult to predict if cyanophycin exists in a soluble or insoluble form in *C. perfringens*. Our experimental findings that spore morphology is altered but heat resistance and germination efficiency are not defective suggest that cyanophycin plays a role in spore assembly, perhaps as a matrix material that gives spores their normal morphology, but its precise role remains to be determined. The presence of cyanophycinase that was associated with spore membranes after germination suggests that spore cyanophycin is degraded after germination is initiated. Given that many species of *Clostridium* related to *C. perfringens* have the cyanophycin/cyanophycinase pair (19) and they are widespread in *Bacillus* species as well (recently determined by using the BLAST program [see http://blast.ncbi.nlm.nih.gov/]), it seems likely that they will have a common function in spore formation.

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