Vegetative Cell and Spore Proteomes of *Clostridioides difficile* Show Finite Differences and Reveal Potential Protein Markers

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Ⓢ *Supporting Information*

ABSTRACT: *Clostridioides difficile*-associated infection (CDI) is a health-care-associated infection caused, as the name suggests, by obligate anaerobic pathogen *C. difficile* and thus mainly transmitted via highly resistant endospores from one person to the other. In vivo, the spores need to germinate into cells prior to establishing an infection. Bile acids and glycine, both available in sufficient amounts inside the human host intestinal tract, serve as efficient germinants for the spores. It is therefore, for better understanding of *C. difficile* virulence, crucial to study both the cell and spore states with respect to their genetic, metabolic, and proteomic composition. In the present study, mass spectrometric relative protein quantification, based on the $^{14}N/^{15}N$ peptide isotopic ratios, has led to quantification of over 700 proteins from combined spore and cell samples. The analysis has revealed that the proteome turnover between a vegetative cell and a spore for this organism is moderate. Additionally, specific cell and spore surface proteins, vegetative cell proteins CD1228, CD3301 and spore proteins CD2487, CD2434, and CD0684 are identified as potential protein markers for *C. difficile* infection.

KEYWORDS: *Clostridioides difficile*, vegetative cells, endospores, proteomes, quantitative proteomics, protein markers
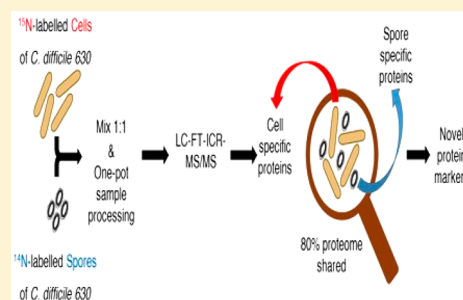
INTRODUCTION

Clostridioides (previously *Clostridium*) *difficile*, an anaerobic, Gram-positive pathogen, is the causative agent of an infection (CDI) characterized by pseudomembranous colitis and nosocomial diarrhea. While an overextensive use of antibiotics has been implicated for the spread of CDI, high rates of asymptomatic colonization make its diagnosis challenging.[1] This necessitates designing strategies and algorithms to optimize the diagnostic tools.[2] Pathogenesis of CDI is manifested via its Rho-glycosylating toxins TcdA and TcdB.[3] Moreover, in response to adverse conditions, *C. difficile* can form endospores—multilayered, highly resistant cellular entities—that are the main transmissible forms[4] in *C. difficile* infections. The spores can germinate in the intestinal environment upon interaction with bile acid mediated by the CspC pseudoprotease,[5] subsequently manifesting the infection.

To date, substantial research has been done on *C. difficile* vegetative cells and spores to understand the survival mechanisms of this pathogen. However, research performed on spores has gained more importance, owing to their crucial role in survival of *C. difficile* in aerobic environments. The number of spore-related genes identified in *Clostridia* is significantly smaller than that in *Bacilli*.[6] Less than 25% of the spore coat proteins of *B. subtilis* have homologues in *C. difficile*, and unlike in *B. subtilis*, in *C. difficile*, neither does

the activation of σ^G rely on σ^E nor is it required for the production and σ^K activation.[7−9] Furthermore, in *C. difficile* spores, cortex hydrolysis occurs before the release of Ca^{2+}-dipicolinic acid complex,[10] whereas in *B. subtilis* spores, the release of this complex precedes cortex hydrolysis.

In the past decade, a few transcriptomic studies,[11,12] quantitative proteomic studies[13−19] and a lipoproteomic study[20] have been done on *C. difficile* vegetative cells. Lawley and colleagues described a protocol to purify spores and performed an extensive proteomic characterization of *C. difficile* 630 spores.[21] Shortly after, we used a gel-free proteomic method and a one-pot sample processing method that focused on the spore surface layers of *B. cereus* and *C. difficile*.[22] Moreover, previous studies have described exosporium removal methods for *C. difficile* spores, and examined the exosporium protein components[23] as well as spore surface glycoproteins.[24] Yet, none of these studies focuses on a comparative analysis that functionally links the spore and vegetative cell proteome. In the present study, we have quantitatively characterized the *C. difficile* vegetative cell proteome relative to that of spores. To this end, spores are mixed with ^{15}N-metabolically labeled vegetative cells based on

spore or cell number, and the mixture is processed with our recently developed one-pot method for mass spectrometric analyses, where the $^{14}N/^{15}N$ isotopic protein ratios represent the relative spore over vegetative cell protein abundances. We aim to deduce putative spore- and vegetative cell-predominant protein markers for *C. difficile*.

■ MATERIALS AND METHODS

Bacterial Strains, Cell Culture, and Sporulation

C. difficile strain 630 (ATCC BAA1382), acquired from the Leibniz Institute of Microorganisms and Cell Cultures, Germany, was used to derive vegetative cells and spores. All cultivations were performed at 37 °C in an anaerobic chamber (Whitley DG250) supplied with a gas mixture comprising 10% hydrogen, 10% carbon dioxide, and 80% nitrogen. The cells were first grown overnight in Schaedler anaerobic broth (Oxoid, CM0497) and further passaged thrice through the newly developed ^{15}N-yeastolate medium (described below) to obtain ^{15}N-labeled vegetative cells. After the third passage, the cells were grown overnight until $OD_{600} \approx 1.7$ and harvested by centrifugation. These cells were then aliquoted and stored at −20 °C until further use. To obtain spores, the vegetative cells were precultured overnight in Columbia broth and inoculated in Clospore medium.[25] Typically, bottles containing 500 mL of Clospore medium, kept in the anaerobic chamber overnight, were inoculated with the precultures. Spores were harvested after 2 weeks of incubation and intensively purified using a combination of ultrasonication, enzyme treatment (lysozyme, trypsin, and proteinase K), and washing with sterile milli-Q water.[21,25] The spore crops were subjected to density gradient centrifugation by layering spores suspended in 20% Histodenz (Sigma-Aldrich, USA) on top of 50% Histodenz in 2 mL Eppendorf tubes, and centrifuging for 25 min at 15 000g.

Preparation of ^{15}N-Yeastolate Medium

Saccharomyces cerevisiae CEN. PK1137D was grown at 37 °C in a defined CBS medium[26] modified with $^{15}NH_4Cl$ (replacing $(NH_4)_2SO_4$) as the sole nitrogen source. Yeast cells were harvested by centrifugation (5000g, 30 min) and washed with water. The protocol to generate yeastolate was adapted from previous studies.[27,28] The yeast cells were made into a 30% (w/v) slurry, ultrasonicated by a tip ultrasonicator for 30 min. The pH of the slurry was adjusted to 7.5 using NaOH before incubating under continuous shaking for 5 days at 50 °C. Thereafter, the slurry was ultrasonicated again and centrifuged at 20 000g for 30 min to collect the supernatant. The pellet was washed twice, and the supernatants were combined and lyophilized, to generate powdered yeastolate. The final yeastolate medium contained 2% ^{15}N-yeastolate, 2% glucose, and 0.2% NaCl.

One-Pot Sample Processing

The one-pot protocol has been previously described in detail.[17] Typically, spores and cells were mixed in 1:1 ratio based on the spore or cell counts and suspended in lysis buffer (6 M urea, 5 mM DTT in 50 mm ammonium bicarbonate buffer at pH = 8.0) and disrupted for seven cycles with 0.1 mm zirconium beads (BioSpec Products, Bartlesville, OK, USA) using a Precellys 24 homogenizer (Bertin Technologies, Aix en Provence, France). The tubes were incubated for 1 h at 56 °C and alkylated using 15 mM iodoacetamide for 45 min at room temperature in the dark. The reaction was quenched with 20 mM thiourea and digestion with Lys-C (at 1:200

protease/protein ratio) was carried out for 3 h at 37 °C. Samples were diluted with 50 mM ammonium bicarbonate buffer and digested with trypsin (at 1:100 protease/protein ratio) was carried out at 37 °C for 18 h. The tryptic digest was freeze-dried. Before use, the freeze-dried samples were redissolved in 0.1% TFA and desalted using Omix μC18 pipet tips (80 μg capacity, Varian, Palo Alto, CA, USA) according to the manufacturer's instructions.

Fractionation of Peptides

ZIC-HILIC chromatography was used to fractionate the freeze-dried peptide samples. Dried digests were dissolved in 500 μL of Buffer A (85% acetonitrile, 5 mM ammonium acetate, 0.4% acetic acid, pH 5.8), centrifuged to remove any undissolved components, and injected into the chromatography system. An isocratic flow with 100% Buffer A for 10 min was followed by a gradient of 0−30% Buffer B (30% acetonitrile, 5 mM ammonium acetate, 0.5% acetic acid, pH 3.8) in the first phase and 30−100% of Buffer B in the second phase (flow rate 400 μL/min). The peptides were eluted and collected in 10 fractions, freeze-dried, and stored at −80 °C until further use.

LC-FT-ICR MS/MS Analysis

ZIC-HILIC fractions were redissolved in 0.1% TFA, peptide concentrations were determined by measuring absorbance at 205 nm and 300 ng tryptic peptide mixtures were injected for analyses. LC−MS/MS data of each ZIC-HILIC fraction were acquired with an Apex Ultra Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 7 T magnet and a Nano electrospray Apollo II Dual Source coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA, USA) HPLC system. LC conditions and acquisition parameters were as described previously.[17]

Data Analysis and Bioinformatics

Each raw FT-MS/MS data set was mass calibrated better than 1.5 ppm on the peptide fragments from the coinjected GluFib calibrant. The 10 ZIC-HILIC fractions were jointly processed as a multifile with the MASCOT DISTILLER program (version 2.4.3.1, 64 bits), MDRO 2.4.3.0 (MATRIX science, London, UK), including the Search toolbox and the Quantification toolbox. Peak-picking for both MS and MS/MS spectra was optimized for the mass resolution of up to 60 000. Peaks were fitted to a simulated isotope distribution with a correlation threshold of 0.7, with minimum signal-to-noise ratio of 2. The processed data were searched in a MudPIT approach with the MASCOT server program 2.3.02 (MATRIX science, London, UK) against the *C. difficile* 630 ORF translation database. The MASCOT search parameters were as follows: enzyme, trypsin; allowance of two missed cleavages; fixed modification, carboamidomethylation of cysteine; variable modifications, oxidation of methionine and deamidation of asparagine and glutamine; quantification method, metabolic ^{15}N labeling; peptide mass tolerance and peptide fragment mass tolerance, 50 ppm. MASCOT MudPIT peptide identification threshold score of 20 and FDR of 2% were set to export the reports.

Using the quantification toolbox, the quantification of the light spore peptides relative to the corresponding heavy cell peptides was determined as light/heavy ratio using Simpson's integration of the peptide MS chromatographic profiles for all detected charge states. The quantification parameters were as
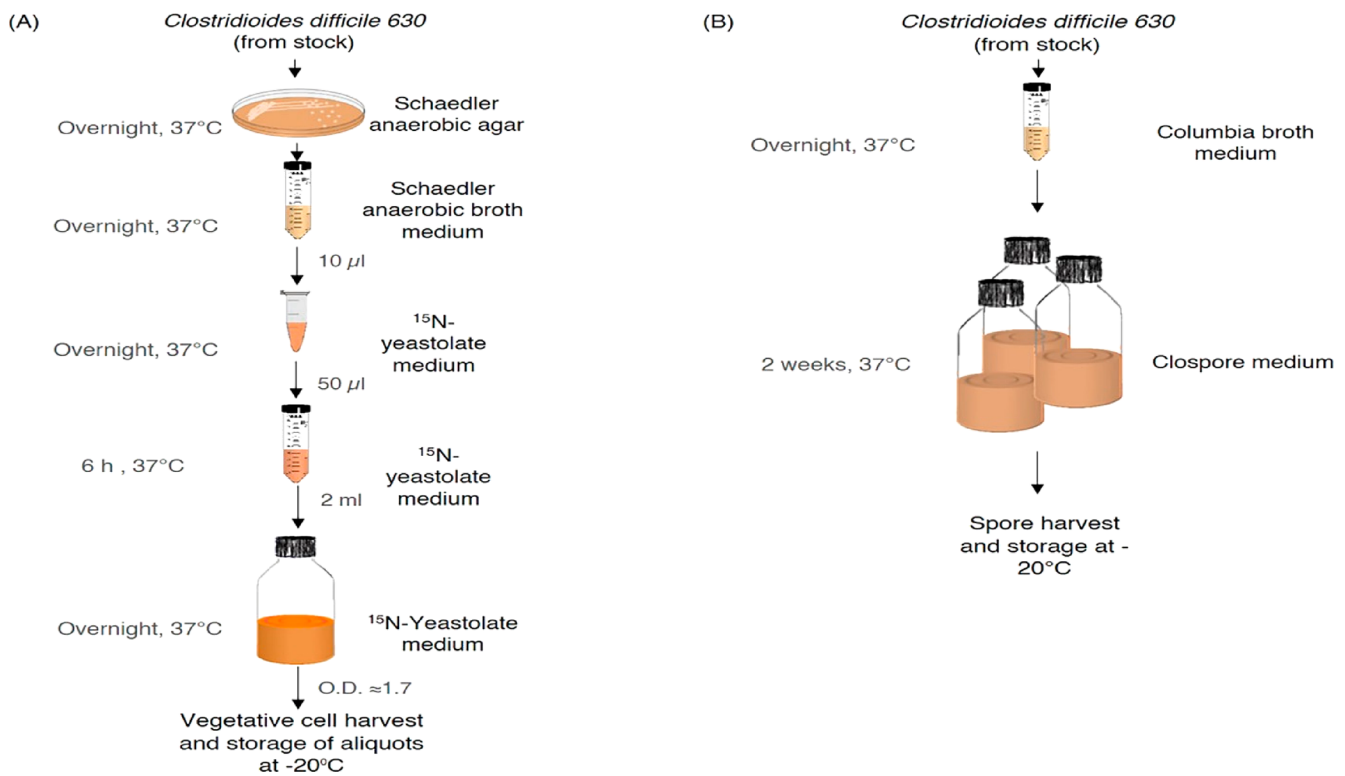
Figure 1. Preparation workflow of (A) ^{15}N-labeled vegetative cells and (B) ^{14}N spores of *C. difficile* 630. See the Materials and Methods section for more details. The images for Petri dish (http://www.clker.com/clipart-red-petri-dish-3.html), media bottle (http://www.clker.com/clipart-reagent-bottle-with-growth-media.html), the Eppendorf tube (https://www.clipartmax.com/middle/m2i8H7m2A0G6N4G6_isop-eppi-pellet-zymo-clip-art-at-clker-eppendorf-tube/), and 50 mL tube (https://openclipart.org/detail/170165/50ml-centrifuge-tube) are obtained from copyright-free public domain Web sites and further modified using Microsoft Power Point 2016.
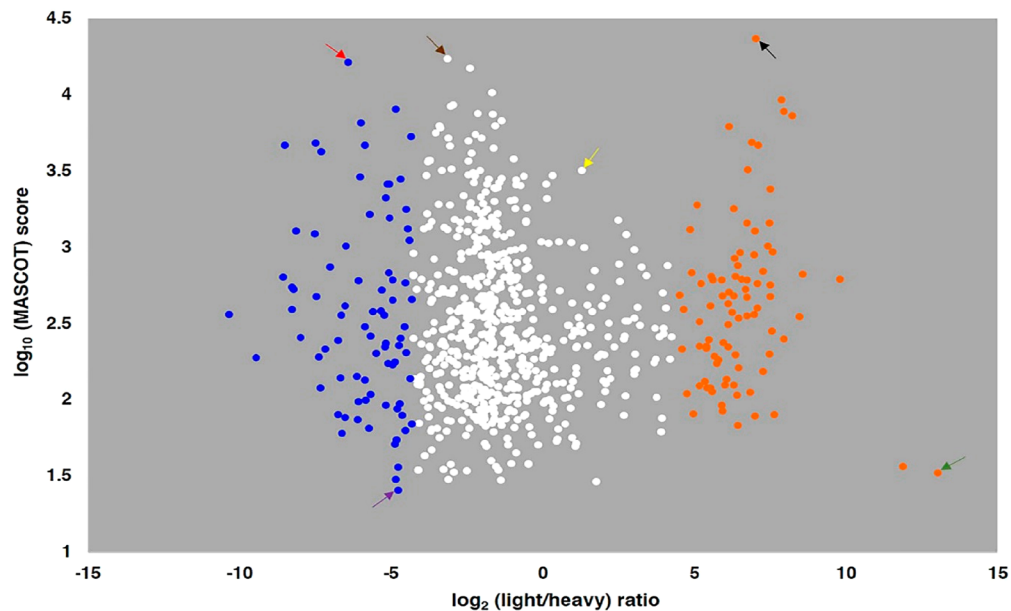

Figure 2. Distribution of proteins in *C. difficile* 630 spores and vegetative cells. MASCOT score indicates the combined spore and cell abundance of a protein versus its light/heavy protein isotopic ratio, which represents the relative level of the protein in spores over vegetative cells. The orange dots indicate spore-predominant proteins (light/heavy ratios > 20), blue dots indicate vegetative cell-predominant proteins (light/heavy ratios < 0.05), and white dots indicate proteins common between spores and vegetative cells (20 > light/heavy ratios > 0.05). Black arrow, SspA; green arrow, CD2657; red arrow, SlpA; purple arrow, CD0594; brown arrow, CD0825; and yellow arrow, CD0718. See the text and Table S1 for more details.

follows: Correlation threshold for isotopic distribution fit, 0.98;

^{15}N label content, 99.6%; XIC threshold, 0.1; all charge states

on; max XIC width, 120 s; elution time shift for heavy and light peptides, 20 s. All isotope ratios were manually validated by inspecting the MS spectral data. The protein isotopic ratios

Table 1. Uniprot Keywords Annotation Enrichment of Quantified *C. difficile* 630 Spore and Vegetative Cell Proteins Based on DAVID Functional Annotation Analysis

UniProt keyword[a]	no. of proteins			UniProt keyword[a]	no. of proteins		
	spore proteome	vegetative cell proteome	commonly shared		spore proteome	vegetative cell proteome	commonly shared
Aminotransferase		10		Cell division	11	13	11
Arginine biosynthesis		7		Flavoprotein	14	12	11
Cell shape		8		FAD	11	10	9
Elongation factor		5		NADP	11	12	11
Peptidoglycan synthesis		7		tRNA-binding	11	11	11
Cytoplasm	130	136	127	NAD	12	11	10
Transferase	115	121	110	Ion transport	10	11	10
Nucleotide-binding	107	114	105	Pyruvate	11	11	11
Hydrolase	107	104	94	Chaperone	10	10	10
ATP-binding	89	95	87	Manganese	10	9	9
Metal-binding	82	80	78	ATP synthesis	9	9	9
Oxidoreductase	61	65	54	Aminopeptidase	9	9	9
Ribonucleoprotein	48	48	48	Lysine biosynthesis	8	8	8
RNA-binding	49	48	48	Hydrogen ion transport	8	8	8
Ligase	46	48	46	Glycolysis	8	7	7
Ribosomal protein	47	47	47	Stress response	7	7	7
Protein biosynthesis	34	36	34	Diaminopimelate biosynthesis	6	6	6
Lyase	31	36	28	Pyrimidine biosynthesis	6	6	6
Magnesium	33	33	33	CF(1)	5	5	5
rRNA-binding	32	32	32	One-carbon metabolism	5	5	5
Zinc	30	28	28	DNA-directed RNA polymerase	4	4	4
Amino-acid biosynthesis	22	27	22	Methionine biosynthesis	4	4	4
Isomerase	25	26	24	Virion	10		
Aminoacyl-tRNA synthetase	23	24	23	Capsid protein	9		
Protease	25	20	20	Rotamase	5		
GTP-binding	15	16	15	Metalloprotease	6		
Pyridoxal phosphate	13	14	12				
Glycosyltransferase	14	13	13				
Cell cycle	11	13	11				

[a]EASE score, i.e., *p*-value threshold for the keyword annotation enrichment, was set to 0.05.

were then calculated as the average over the corresponding peptide ratios. For each of the three replicas, the identification and quantification reports were imported into a custom-made program to facilitate data combination and statistical analysis. Protein identification was validated with identifications in at least two replicas. For these identified proteins the relative quantification was calculated as the geometric mean of at least two validated light/heavy ratios. All identification and quantification protein data are listed in Supplementary Table S1. The mass spectrometry proteomics data have been deposited as a partial submission to the ProteomeXchange Consortium via the PRIDE[29] partner repository with the data set identifier PXD012030.

Transmembrane proteins were predicted using the default parameters on the TMHMM Server (version 2.0 http://www.cbs.dtu.dk/services/TMHMM/). DAVID Bioinformatics Resources tool (version 6.8) was used[30] to retrieve the functional annotation data of UniProt keyword and KEGG pathway classifications. The BioCyc pathway analysis tool[31] was used to generate a cellular overview of the quantified proteins.

■ RESULTS

Metabolic Labeling of *C. difficile* Vegetative Cells Using ^{15}N-Yeastolate

As illustrated in Figure 1 our culturing methods successfully yielded ^{15}N labeled vegetative cells and ^{14}N spores. For a

number of identified ^{15}N labeled vegetative cell peptides, the ^{15}N label content has been calculated on the basis of their mass spectrometric isotope patterns using the NIST isotope calculator.[32] This shows that the present metabolic labeling method achieves a ^{15}N label content of $\geq$99.5%, which is amply sufficient for accurate protein quantification.

Identification and Quantification of Cell and Spore Proteins

A total of 1095 proteins has been identified from *C. difficile* spores and vegetative cells of which 796 have been relatively and reproducibly quantified between spores and vegetative cells (Supplementary Table S1). Figure 2 represents a distribution of quantified proteins, where the abundance of the combined spore and vegetative cell proteins indicated by the $\log_{10}$ values of their MASCOT scores are plotted against the corresponding relative protein levels in the two morphotypes indicated by the $\log_2$ values of the light/heavy ratios. Eighty seven proteins are considered to be predominantly present in spores with a light/heavy ratio >20, while 81 proteins are considered to be predominant present in vegetative cells with light/heavy ratios <0.05. From the remaining 628 commonly shared proteins, 18% are enriched in spores with light/heavy ratio between 1 and 20, while 82% are enriched in cells with a light/heavy ratio between 1 and 0.05. In total, 167 proteins have been classified as membrane proteins by the TMHMM analysis (Supplementary Table S2).
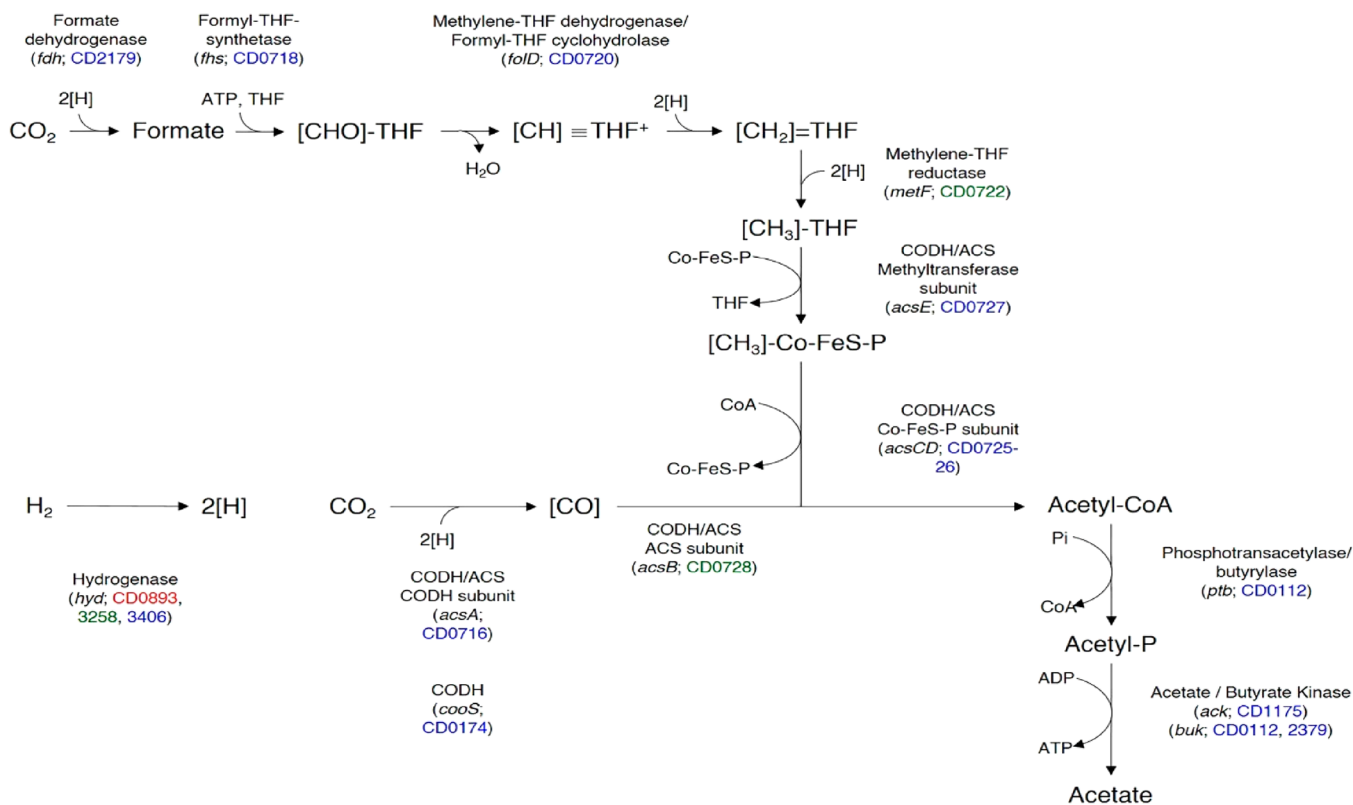
Figure 3. Classification of proteins associated with the Wood−Ljungdahl pathway identified in *C. difficile* 630. Proteins presented in red, green, and blue fall under the categories of vegetative cell-predominant, spore-predominant, and shared proteins, respectively.

The cellular overview based on pathway analysis of the quantified proteins is represented in Supplementary Figure S1 and S2 indicating the pathways to which these commonly shared proteins belong.

Spore-Predominant Proteins

These include proteins from the spore coat and exosporium layers, classified under UniProt Keywords virion and capsid proteins, along with some rotamase proteins and metalloproteases (Table 1). SspA is the most abundant protein in this category, whereas an uncharacterized protein CD2657 (with 13 times higher levels in spores than in vegetative cells) is the least abundant (Figure 2). It is noteworthy that for most of the proteins in the spore-predominant category, their corresponding genes are upregulated during sporulation (see Supplementary Table S3). The TMHMM analysis classified 26 proteins from this category as membrane proteins (Supplementary Table S2). Most of these are uncharacterized membrane proteins but some are known proteins such as SpoVD, SpoVAC, SpoVFB, FtsH, and DacF.

Cell-Predominant Proteins

These include the cytoplasmic proteins such as aminotransferases, arginine biosynthesis, elongation factors, cell shape and peptidoglycan synthesis proteins (Table 1). The surface layer protein SlpA is the most abundant and unique protein in this category, whereas an uncharacterized protein CD0594 (with levels 27 times higher in vegetative cells than in spores) is the least abundant (Figure 2). Eighteen membrane proteins are predominant in vegetative cells, as predicted by TMHMM (Supplementary Table S2).

Proteins Shared between Spores and Vegetative Cells

The proteins shared between spores and cells are mostly ribosomal proteins, cell cycle-regulating and/or associated proteins, and cytosolic proteins involved in pathways required for anabolism and catabolic pathways of energy metabolism distributed over 46 categories by DAVID (Table 1, Supplementary Figure S1 and S2). These also include products of 25 essential genes[33] such as peptidoglycan synthesis protein MurG (CD2651) and formate-tetrahydrofolate ligase CD0718 (2 and 2.4 times higher levels in spores than in vegetative cells, respectively), and *S*-adenosylmethionine synthase MetK as well as a rubrerythrin CD0825 (~4 and ~9 times higher levels in vegetative cells than in spores, respectively) (Figure 2). The TMHMM analysis of the shared proteins has identified 123 membrane proteins (Supplementary Table S2), such as the phosphotransferase system (PTS) of sugar transporters, ABC-type transporters, and V-type ATPases. These also included most proteins involved in the Wood−Ljungdahl pathway (Figure 3). From Table 1 it can be deduced that proteins from this category that are present in spores are essentially those that are required for hibernation, the initiation of growth, and the resumption of metabolism upon germination and outgrowth.

◼ DISCUSSION

To our knowledge, the vegetative cell and spore proteomes of *C. difficile* have been explored for the first time in a single experimental setup to understand the fundamental differences between these two morphological forms of this obligate anaerobic bacterium. To this end, the spores and ^{15}N-labeled vegetative cells have been mixed for relative protein quantification. Metabolic labeling using the ^{15}N isotopes is a

highly accurate means of proteome quantification.[34] However, a method for labeling *C. difficile* was unavailable until recently.[19] Here, another method for metabolic labeling using ^{15}N-labeled yeastolate medium is presented, which provides a simple, economical, and rapid means to perform quantitative proteomics of a variety of pathogenic and nonpathogenic microbes. Our analyses show that 80% of the quantified proteins are common to both the cells and the spores, indicating that the pathogenic *C. difficile* employs a relatively modest proteomic changeover to enable a long-term survival as a dormant spore. The corresponding pathways are shared between the vegetative cells and spores (Supplementary Figure S1 and S2) however the relative quantities of these proteins vary between the two distinct cellular entities. A discussion of quantified and functionally key proteins, that indicate the differences in the two morphotypes of *C. difficile*, is presented below.

C. difficile expresses an array of cell surface proteins, including the S-layer proteins (such as SlpA) and its paralogues from the cell wall protein (CWP) family, for adherence and/or colonization. In this study, 8 CWP family proteins have been quantified: Cwp18 and 22 being identified in both morphotypes with higher levels in spores, whereas Cwp2, 5, 6, 19, 84, and CwpV being identified in vegetative cells. Cwp22, the LD-transpeptidase (Ldt$_{cd2}$), is an important protein that plays a role in peptidoglycan remodelling[35] and plausibly confers resistance to β-lactam antibiotics.[36] In fact, recently, Cwp22 has also been shown to be involved in toxin production, sporulation, motility, and cell viability in *C. difficile* strain R20291.[37] CwpV promotes *C. difficile* aggregation and its strain-dependent structural variations may assist in evading the host antibody response[38] or to launch an antiphage strategy.[39] Disseminating spores, on the contrary, make use of the hydrophobic exosporium to adhere to and colonize surfaces. Differing with the spores of *Bacillus* spp., such as the *B. anthracis* spores,[40] the *C. difficile* spores are not reported to possess the interspace region between the spore coat and the fragile, heterogeneous exosporium.[41−43] Although most known and putative exosporium proteins described previously[23] have been identified in this study, the BclA family proteins have not (except BclA1 encoded by CD0332, identified in one replicate and thus not quantified). An absence of hair-like structures in the *C. difficile* 630 exosporium[43] or the loss of exosporium due to the sonication and protease treatment earlier during spore purification[44] may underlie this finding. Other identified proteins such as CD1474, CD2845, and CD1524—all rubrerythrins—likely present in the exosporium, may play a role in fighting reactive oxygen species and oxidative stress.[45] The "stay-green" family protein CD3613 is also a putative exosporium protein. Usually, the proteins belonging to this family are involved in chlorophyll degradation,[46] but the upregulation of *cd3613* during sporulation in a mouse model[11] suggests a potential role in transmission. The quantified spore envelope protein CD2635 is previously suggested to be involved in germination.[12] This protein, similar to CD2636, contains a characteristic YIEGIA domain and both could play significant roles in spore assembly as well as disintegration.

C. difficile relies heavily on the phosphoenolpyruvate-dependent phosphotransferase system (PTS) for uptake and regulation of various sugars and sugar derivatives.[47] The PTS is clearly advantageous for germinating spores, since various sugars are readily available in the human gut and may thus be used to facilitate outgrowth and establish infection.[48,49] We have identified 22 PTS proteins, of which 15 have been quantified (Supplementary Table S1). The quantified PTS proteins are shared between the vegetative cells and spores with an exception of CD3027, which appears to be specific for the vegetative cells. Along with HPr (PtsH/CD2756) and Enzyme I (PtsI/CD2755), the quantified PTS proteins function in the transport of fructose (FruABC/CD2269, CD2486−88), glucose (PtsG/CD2667, CD3027, CD3089), mannitol (MtlF/CD2332 and MtlA/CD2334), mannose (CD3013−14), xyloside (XynB/CD3068), and β-glucoside (BglF5/CD3137). Although shared, CD3013−14, CD2486−88, CD3089, PtsH, and MtlA-F show relatively higher levels in spores, whereas PtsG, PtsI, BglF5, FruABC, and XynB show lower levels in spores. In line with these observations, a previous study has shown that in germinating spores, *bglF5* and *ptsG* transcripts are downregulated, whereas those of *fruABC* and *cd2486-87* are upregulated.[50] In mouse infections, *cd2487* is upregulated 14 h postinfection, whereas proteins XynB, CD3027, and PtsI are seen to be downregulated 38 h postinfection.[11] In pig infections of *C. difficile*, PtsI, BglF5 (4−12 h postinfection) and MtlA (only 12 h postinfection) are upregulated[51] and XynB and CD3013−14 are downregulated. Furthermore, MtlA and MtlF can repress *tcdA* and *tcdB* genes and thus toxin expression in *C. difficile*.[52] Put together, these studies indicate that PtsI, BglF5, CD2487, and MtlA potentially play a role in the pathogenesis of *C. difficile* infections; CD2487 and MtlA are predominantly present in spores, making them important targets in understanding spore persistence.

Non-PTS transport systems are also involved in carbohydrate uptake in Clostridia.[47] In this respect, of all ATPases and related proteins quantified, only 6 and 4 proteins belong to the cell-predominant and spore-predominant categories, respectively. Four V-type ATPases have been quantified from spores of which, only AtpC is spore-predominant whereas the other three are shared with cells. Nevertheless, their presence in spores highlights their role along with the Rnf complex proteins (present at lower levels in spores), in ATP synthesis.[53] AtpC is associated with proton transport, possesses a hydrolase activity, and contains a CodY-binding region,[54] thus potentially repressing toxin expression[55] and regulating synthesis as well as circulation of pyruvate and 2-oxoglutarate in the cell[56] to provide a proteomic flexibility during *C. difficile* spore revival. The spore-predominant ATPases also include a cation (Ca^{2+})-transporting ATPase (CD2503), which shares 42% identity with *B. subtilis* YloB ATPase, likely responsible for accumulating intracellular calcium and reinforcing thermal resistance.[57] Peculiarly, two ABC-type transporters—lipoproteins CD2365 and SsuA, also identified in the spore inner membrane (our unpublished results)—are shared vegetative cells and spores[20] but are present at higher levels in spores. These are alkanesulfonate and taurine binding proteins, respectively and SsuA is also involved in sulfur metabolism.[58] Interestingly, the taurine side chain of taurocholate selectively binds its potential receptor site[59] and taurine itself is an alkanesulfonate, thus higher levels of SsuA and CD2365 in spores could indicate potential taurine interaction during germination. Identified ATP/GTP binding protein CD0114 bears 25% identity with protein CD3298, a protein associated with spore germination,[60] making it a worthy target for spore germination studies.

Amino acids play a crucial role in spore germination and functioning of the Stickland pathways in *C. difficile* cellular

physiology. Therefore, amino acid transporters are also important for germinating spores as they transform into vegetative cells. Proteins CD3458 and CD1555 are putative amino acid permeases identified to be slightly more abundant in spores than in vegetative cells. In addition, protein CD3458 contains a putative amino acid permease domain and an SLC5−6-like_sbd superfamily domain, thus qualifying as an amino acid permease and sodium/glucose cotransporter. Another amino acid permease CD2612, although identified in vegetative cells, is upregulated in the presence of cysteine,[58] implying a role in sulfur metabolism. Cell-predominant protein CD2344 contains an Asp-Al_Ex domain found in aspartate-alanine antiporters and might be capable of developing a membrane potential enough to carry ATP synthesis via FoF1 ATPase.[61]

From the quantified data set, 198 proteins are classified to the metabolic pathways category by DAVID analysis. Although the spores are metabolically dormant, the proteins belonging to the amino acid biosynthesis, purine metabolism, glycolysis, fatty acid metabolism, and nitrogen metabolism are present and form the core protein set in spores. Moreover, in spores, arginine biosynthesis pathway proteins are present at ∼20% of the levels detected in vegetative cells. This indicates that germinating spores require de novo synthesis of these proteins postgermination to assist the outgrowing cells. Ribosomal proteins—except the 50S ribosomal protein L30 (CD0881)—are also present in low amounts in *C. difficile* spores. CD0881 has a ferredoxin-like fold, resembling the structure of yeast L7 proteins, and is likely involved in processing precursors of large rRNAs,[62] a function that could well aid the outgrowing spores. The phosphate butyryltransferases (CD0715 and CD0112/Ptb) involved in the butanoate metabolism pathway are present not only in vegetative cells but also in spores, thus conferring on the spores a metabolic flexibility. *C. difficile* may also deploy several sulfur and nitrogen metabolism proteins while surviving in anaerobic conditions. Of these, only CD2431, a nitrite/sulfite reductase, is abundant in spores. This protein also contains a 4Fe-4S domain and can catalyze the reduction of sulfite to sulfide and nitrite to ammonia.[63]

C. difficile and other acetogenic Clostridia have acquired such metabolic flexibility that they can directly utilize the CO_2 and H_2 from air and yield a variety of products including acetate and methane.[64] This Wood−Ljungdahl pathway of acetogenesis is believed to be the first biochemical pathway to have emerged on earth[65] and all proteins involved in this pathway are identified in *C. difficile* 630, which reinforces the acetogenic nature of *C. difficile* growth. Of these, CD3405, CD3407, and CD0730 have been detected only in single replicates and thus are not quantified. The other Wood−Ljungdahl pathway proteins have all been quantified, with only three proteins—MetF (CD0722), CD0728, and CD3258—being highly abundant in spores. In contrast, only a single protein—CD0893—is predominant in vegetative cells. The acetogenic mode of life of *C. difficile* requires specific enzymes, such as acetyl-CoA synthases/CO dehydrogenases (CD0174, CD0176, and CD0727), formate dehydrogenases (CD2179), and iron-only hydrogenases (CD0893, CD3258, and CD3406). Enzymes CD0174 and CD0176 synthesize the key metabolite acetyl-CoA from CO, methyl corrinoid, and CoASH. The formate dehydrogenases can be seleno (CD3317) or nonseleno (CD0769 and CD2179) enzymes. Protein CD2179, an anaerobic dehydrogenase, reduces CO_2 to formate, which is further metabolized to acetyl-CoA through

enzymatic reactions. One of these enzymes, CD0727, is a unique acetyl-CoA synthase/CO dehydrogenase with a methyltransferase subunit and is reported to be essential for sporulation.[50] The acetogens lacking cytochromes use the Rnf complex (encoded by CD1137−42) discussed above as the putative coupling site for energy conservation.[64] In the present study, all components of the Rnf complex, except CD1140−41, have been identified and have low levels in spores. The Rnf complex proteins, together with electron transport flavoproteins etfA2/B2 (CD1055−56), are employed in butyrate formation.[66] However, the present study has identified only etfA1/B1 and etfA3/B3 proteins. These proteins are predominant in vegetative cells, indicating that they likely function exclusively during the vegetative life cycle of *C. difficile*.

The iron-only hydrogenases are 10 times more efficient in hydrogen production than [NiFe] hydrogenases[67] and are abundant in Clostridia. *C. difficile* encodes two trimeric and three monomeric hydrogenases.[68] Proteins CD3405−3407 function as electron-bifurcating hydrogenases whereby physiological electron carriers such as ferredoxin are used for H_2 production.[69] In the present study, CD3258 is seen predominantly in spores, whereas CD0893 occurs mostly in vegetative cells. Both proteins are monomeric, ferredoxin dependent,[69] and contain a H-cluster, i.e., a center for hydrogen production.[70] However, CD3258 has a sequence of eight cysteines for stabilizing two [4Fe4S] clusters transferring electrons from the surface to the protein's active site[71] whereas CD0893 has a single FeS domain with a $(Cx_{1-4}Cx_{5-9}Cx_3C)$ arrangement at its N-terminus and the H-cluster has an additional cysteine residue (TSCCCPxW[68]). The predominant expression of CD3258 and CD0893 in spores and vegetative cells, respectively, indicates the distinct roles of these proteins in *C. difficile* physiology.

Peptidases and proteases are crucial for various cellular processes and spore formation as well as germination. In this study, 29 peptidases and 12 proteases are identified and quantified. These include spore-specific proteins involved in germination, such as Gpr (CD2470) and CspBA. In the present study, CspC pseudoprotease has been detected in only one replicate and thus not quantified. Other proteins involved in cellular regulatory processes, such as ATP-dependent Clp proteases, zinc metalloproteases, serine proteases, Lon proteases, have also been quantified in the present study. These peptidases belong to various families such as aminopeptidase (M1, M18), metalloendopeptidase (M16), membrane dipeptidase (M19), glutamate carboxypeptidase (M20), glycoprotease (M22), methionyl aminopeptidase (M24), prolyl oligopeptidase (S9), and collagenase (U32). It is speculated that proteins belonging to the M22 and U32 family (CD0150 and CD1228, respectively) function in spore germination. In fact, BA0261 from *B. anthracis*, an orthologue of protein CD0150 shared by *C. difficile* cells and spores, is suggested to play a role in spore germination[72] and a collagenase is implicated in virulence of *B. cereus* endophthalmitis[73] indicating a similar potential for CD1228. A previous study suggested that a Lon protease in *Brucella sp.* is involved in BALB/c mice infections.[74] Thus, the Lon protease CD3301, present in vegetative cells and spores, may play a role in infection. Protein CD0684, present in *C. difficile* spores under σ^G regulation, is suggested to be involved in stress resistance.[12] Notably, none of the peptidases or proteases quantified in the present study are specific to vegetative cells.

Finally, there are also a few quantified but uncharacterized proteins that are worth discussing. For instance, protein CD2434 has a UBA_NAC_like bacterial protein domain commonly found in proteins involved in ubiquitin-dependent proteolysis.[75] A previous study has shown that the *E. coli* toxin CNF1 utilizes the ubiquitin-proteasome assembly of host cells to partially inactivate their Rho GTPases,[76] a mechanism similar to that of TcdA and TcdB toxins.[77] Thus, although not a direct evidence, this observation suggests involvement of CD2434 in pathogenesis. Contradicting the previous reports,[78] CD3669—identified as the orthologue of *B. subtilis* GerM— might be involved in sporulation. Additionally, protein CD1319, an orthologue of YlxY, may also be important for sporulation as seen in *B. subtilis*.[79] Protein CD1470, a sulfotransferase, may be involved in cyanide detoxification. Recently, PdaA1 (CD1430) and PdaA2 (CD2719) have been shown to be important for cortex muramic acid-δ-lactam synthesis and spores lacking them are heat sensitive, deficient in germination, and exhibit late virulence.[80] Although CD2719 is not identified in the present study, CD1556, an orthologue of PdaA2 from *B. cereus* var. *anthracis*, is identified. Thus, CD1556 may be important for spore structure and germination or could be a complementary functional paralogue.

CONCLUSIONS

The one-pot sample processing method along with ^{15}N metabolic labeling has enabled a reproducible, combined cell and spore quantitative proteome analysis of the anaerobic pathogen *C. difficile* 630. The analysis outlines a relatively modest proteomic adaptation of this evolutionarily and clinically important anaerobic pathogen, when as a survival strategy, it completes spore formation. In addition to the predominant cell- and spore-surface proteins, the study has qualified shared vegetative cell proteins CD1228, CD3301 and shared spore proteins CD2487, CD2434 as potential protein markers for *C. difficile* infections.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.9b00413.

Figures S1 and S2 (PDF)

Tables S1−S3 (XLSX)

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‖S.B. and C.G.d.K. contributed equally to this work. W.R.A analyzed the data, prepared the figures and tables, and wrote the main manuscript text. L.Z. performed the experiments. L.d.K. conceptualized and designed the experiments as well as curated and processed the proteomics data. S.B. and C.G.d.K. supervised and mentored the research. All authors reviewed the manuscript.

Notes

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