

Transcriptional Profile during Deoxycholate-Induced Sporulation in a *Clostridium perfringens* Isolate Causing Foodborne Illness

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ABSTRACT

Clostridium perfringens type A is a common source of foodborne illness (FBI) in humans. Vegetative cells sporulate in the small intestinal tract and produce the major pathogenic factor *C. perfringens* enterotoxin. Although sporulation plays a critical role in the pathogenesis of FBI, the mechanisms inducing sporulation remain unclear. Bile salts were shown previously to induce sporulation, and we confirmed deoxycholate (DCA)-induced sporulation in *C. perfringens* strain NCTC8239 cocultured with human intestinal epithelial Caco-2 cells. In the present study, we performed transcriptome analyses of strain NCTC8239 in order to elucidate the mechanism underlying DCA-induced sporulation. Of the 2,761 genes analyzed, 333 were up- or downregulated during DCA-induced sporulation and included genes for cell division, nutrient metabolism, signal transduction, and defense mechanisms. In contrast, the virulence-associated transcriptional regulators (the VirR/VirS system, the *agr* system, *codY*, and *abrB*) were not activated by DCA. DCA markedly increased the expression of signaling molecules controlled by Spo0A, the master regulator of the sporulation process, whereas the expression of *spo0A* itself was not altered in the presence or absence of DCA. The phosphorylation of Spo0A was enhanced in the presence of DCA. Collectively, these results demonstrated that DCA induced sporulation, at least partially, by facilitating the phosphorylation of Spo0A and activating Spo0A-regulated genes in strain NCTC8239 while altering the expression of various genes.

IMPORTANCE

Disease caused by *Clostridium perfringens* type A consistently ranks among the most common bacterial foodborne illnesses in humans in developed countries. The sporulation of *C. perfringens* in the small intestinal tract is a key event for its pathogenesis, but the factors and underlying mechanisms by which *C. perfringens* sporulates *in vivo* currently remain unclear. Bile salts, major components of bile, which is secreted from the liver for the emulsification of lipids, were shown to induce sporulation. However, the mechanisms underlying bile salt-induced sporulation have not yet been clarified. In the present study, we demonstrate that deoxycholate (one of the bile salts) induces sporulation by facilitating the phosphorylation of Spo0A and activating Spo0A-regulated genes using a transcriptome analysis. Thus, this study enhances our understanding of the mechanisms underlying sporulation, particularly that of bile salt-induced sporulation, in *C. perfringens*.

Clostridium perfringens, a Gram-positive spore-forming anaerobic bacterium, is highly prevalent in the intestinal tracts of humans and animals and also in nature, such as in soil, sewage, and rivers (1, 2). This bacterium has been identified as a major pathogen in foodborne illness (FBI) and consistently ranks among the most common bacterial FBI in the United States, United Kingdom, and Japan (1, 3, 4). Therefore, controlling this FBI is a critical issue from a public health perspective. FBI occurs when contaminated food materials are undercooked and/or held at improper temperatures, thereby allowing heat-resistant spores to germinate and grow (5). After the ingestion of contaminated foods, vegetative cells sporulate in the small intestinal tract (6). During sporulation, bacterial cells produce *C. perfringens* enterotoxin (CPE), which binds to its receptor on epithelial cells, damages the intestinal epithelium, and results in diarrhea and abdominal cramping (7–11). Thus, the sporulation of *C. perfringens* is a key event for its pathogenesis. Since bacteria generally differentiate or sporulate in response to environmental factors, these factors need to be clarified and the underlying mechanisms regulating this process at the infection site elucidated. However, the factors triggering *C. perfringens* sporulation *in vivo* currently remain unclear.

Bile salts are major components of bile and are secreted from

the liver for the emulsification and solubilization of lipids (12, 13). Primary bile salts, which are composed mainly of cholate and chenodeoxycholate, are synthesized in the liver from cholesterol and conjugate with either glycine or taurine before they are secreted. They can be modified to form secondary bile salts (deoxycholate [DCA] and lithocholate) by intestinal bacterial enzymes. Most secreted bile salts are absorbed by passive diffusion along the entire gut and by active transport in the distal ileum (12). Bile salts are well-known regulatory molecules for the activation of nuclear

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receptors and cell-signaling pathways in mammalian cells (14). Previous studies showed that enteric pathogens possess machineries for resistance to bile in order to survive in the gastrointestinal tract (15, 16) and use bile as an environmental signal for their invasion into the host (17–20). Since *C. perfringens* has inherent bile tolerance (21), bile salts may be used as enteric environmental cues for *C. perfringens* in order to establish infection in the intestinal tract. Bile and bile salts have been shown to accelerate sporulation in *C. perfringens* strains (22–24). We recently confirmed DCA-induced sporulation in *C. perfringens* strain NCTC8239 cocultured with human intestinal epithelial Caco-2 cells (25). However, the mechanisms underlying bile salt-induced sporulation have not yet been clarified.

Morphogenesis and gene regulation during spore formation have been extensively investigated for *Bacillus* spp. (26, 27). Entry into the sporulation pathway is governed by Spo0A, a member of the response regulator family. Spo0A is phosphorylated via a phosphorelay comprised of several kinases and intermediaries (Spo0F and Spo0B) in predivisional cells (sporulation stage 0) to become active. Spo0A~P dimerizes and promotes the transcription of genes involved in activating downstream molecules, including the forespore- and mother cell-specific sigma factors σ^F and σ^E , respectively (stage II). The proteins regulated by these sigma factors in turn control inter- and intracompartment communication between the prespore and/or mother cell during the engulfment and formation of protoplasts (stage III). After the spore cell wall, cortex, and coat have formed (stages IV to V), the spore matures (stage VI) and then is released from the lysed mother cell (stage VII). Transcriptional analyses revealed that the sporulation cascades in clostridia share many common features with those in *Bacillus* spp. (28–32); however, many differences also have been identified. For example, the genes for Spo0F and Spo0B involved in the Spo0A phosphorelay system in *Bacillus* spp. are hardly conserved in clostridia (33); thus, Spo0A appears to be directly phosphorylated by histidine kinases in several clostridia (34–36). The conserved gene products involved in sporulation, such as oligopeptide permeases, have different functions in bacilli and clostridia (37). Furthermore, Xiao et al. (32) recently revealed previously unidentified putative sporulation genes in *C. perfringens* FBI strain SM101 using a whole-genome microarray analysis during its sporulation process. Thus, the detailed mechanism underlying sporulation in clostridia remains unclear. In the present study, we performed a transcriptome analysis on *C. perfringens* FBI strain NCTC8239 during DCA-induced sporulation in an attempt to identify genes involved in a sporulation cascade specifically activated by DCA. We then compared the identified candidate genes with the findings of previous transcriptome analyses during the sporulation of clostridia and bacilli (29–32, 38) and classified common features and DCA-specific responses. It is noteworthy that the phosphorylation of Spo0A was facilitated in the presence of DCA, leading to the upregulation of several Spo0A-controlled genes. The possible mechanisms regulating the DCA-induced sporulation of *C. perfringens* are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The type A FBI outbreak strain *C. perfringens* NCTC8239 was purchased from the National Collection of Type Cultures. *C. perfringens* type A strain SM101 (an electroporatable derivative of FBI strain NCTC8798) was kindly provided by Tohru Shimizu (Kanazawa University). The *spo0A* null mutant of SM101, IH101,

was prepared as described previously (39). In order to achieve sporulation, bacteria were inoculated into fluid thioglycolate (FTG; BD, Franklin Lakes, NJ) medium and incubated anaerobically in an anaerobic jar (AnaeroPack system; Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 18 h. One milliliter of the bacterial culture was passaged into 10 ml Duncan-Strong (DS) medium (40) and cultured at 37°C for 24 h. One milliliter of the culture then was heated at 75°C for 20 min, passaged into 10 ml of fresh DS medium, and incubated at 37°C for 24 h. The heat treatment and passages were repeated until the number of spores observed by phase-contrast microscopy was greater than half the total number of bacteria (25). These bacterial cells were heated and stored at –80°C with glycerol (final concentration, 20%) for future use.

Coculture study. Bacteria were cocultured with human intestinal epithelial Caco-2 cells as described previously (25), but with slight modifications. Briefly, Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Cells were seeded on a 24-well plate (1.3×10^5 cells per well) and incubated for 4 days. Just before the inoculation of bacteria, Caco-2 cells were washed using phosphate-buffered saline (PBS) three times and incubated in 1 ml glucose-negative DMEM [DMEM(–); Life Science Technologies, Carlsbad, CA] supplemented with 0.4% starch and 50 μ M DCA (Wako, Osaka, Japan). NCTC8239 was precultured in FTG anaerobically at 37°C for 18 h. Cultures were washed with PBS twice, and 100 μ l of the bacterial culture (1×10^7 to 5×10^7 CFU per ml) then was inoculated into Caco-2 cells and incubated in the CO₂ incubator at 37°C. The number of viable vegetative cells was determined by plating serially diluted samples onto brain heart infusion (BHI) agar, incubating at 37°C for 24 h under anaerobic conditions, and calculating CFU. The number of heat-resistant spores was counted by plating heat-treated cultures onto BHI agar. The detection threshold was 200 CFU/ml.

Fluorescence staining. Bacterial cultures were centrifuged at $400 \times g$ for 5 min, and the pellets were double stained with rhodamine B (Life Science Technologies) and Hoechst 33342 (Life Science Technologies) for cell membrane and double-stranded DNA, respectively (41). Stained cells were observed under a phase-contrast microscope with fluorescence accessories (Nikon ECLIPSE 80i; Nikon, Tokyo, Japan).

RNA extraction. Total RNA was extracted as described previously (25) but with slight modifications. Bacterial cultures containing 2×10^7 CFU of *C. perfringens* with detached Caco-2 cells were treated with RNAProtect bacterial reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After centrifugation at $5,000 \times g$ for 10 min, the pellets were washed with SET buffer (25% sucrose, 50 mM EDTA [pH 8.0], and 50 mM Tris-HCl [pH 8.0]) at $5,000 \times g$ for 10 min. The pellets, which were suspended in GTC buffer (4 M guanidine thiocyanate, 0.5% Na N-lauryl sarcosine, 25 mM sodium citrate [pH 7.0], and 0.1 M β -mercaptoethanol), were homogenized by being passed three times through a 21-gauge needle to disrupt Caco-2 cell membranes. After centrifugation at $5,000 \times g$ for 10 min to recover the bacterial cells, the pellets were washed with SET buffer once. The bacterial cells were lysed by being suspended in 100 μ l SET buffer with 20 mg/ml lysozyme (Sigma) and 100 μ g/ml proteinase K (Roche Applied Science, Upper Bavaria, Germany) at 37°C for 30 min. After this incubation, they were transferred into a tube containing zirconia beads (Easy Beads; AMR, Gifu, Japan), vortexed for 5 min, and centrifuged at $21,130 \times g$ for 5 min. Total RNA was extracted from the supernatants using TRI Reagent LS (Sigma) according to the manufacturer's instructions.

Custom chip design. Hassan et al. (42) previously reported that NCTC8239 encoded 3,068 genes that translated to 2,784 proteins. From the NCBI database, we acquired 2,778 coding sequences from the 55 contigs of NCTC8239 (GenBank accession number [ABDY000000000.1](http://www.ncbi.nlm.nih.gov/nuccore/NZ_ABDY000000000.1); http://www.ncbi.nlm.nih.gov/nuccore/NZ_ABDY000000000.1) (see Fig. S1 in the supplemental material). We designed a DNA probe utilizing eArray provided by Agilent Technologies (Santa Clara, CA) (<http://www.chem-agilent.com/contents.php?id=29443>) with these coding sequences. We acquired 2,778 probes with information for probe length, melting point

TABLE 1 Primers

Primer name	Primer sequence	Gene target or function	Product size (bp)	Reference
PrMY147	5'-AACAGATTTAGTTGTATTAGCTATAATTATGCC-3'	Mutagenesis		
PrMY145	5'-TAATACAACATAAATCTGGTTGTTTTCTTC-3'	Mutagenesis		
PrMY67	5'-CATGCTATAGAGGTTGCTTG-3'	<i>spo0A</i>	98	
PrMY68	5'-GAGTTTGTGGTTTACCCTT-3'	<i>spo0A</i>		
PrMY73	5'-TGGATAGTTCAGGAATTGGTGTT-3'	<i>spoIIAA</i>	162	
PrMY74	5'-GAGCTTCATCCAAATTTTCGT-3'	<i>spoIIAA</i>		
PrMY71	5'ATGAAGGATTTGCAAGGGTG-3'	<i>spoIIAB</i>	100	
PrMY72	5'-ACAGCCTCAGAAACAGCAGTC-3'	<i>spoIIAB</i>		
PrMY50	5'-AGCCTTAGAGTCAGCAAGTAGT-3'	<i>sigF</i>	118	
PrMY51	5'-ACCATGTCATCATCTTCACCCT-3'	<i>sigF</i>		
PrMY75	5'-TACTGGTGTAGGAGTTGAGG-3'	<i>sigE</i>	111	
PrMY76	5'-CTTGATGCATAGGTTGCAAG-3'	<i>sigE</i>		
PrMY22	5'-CGTTCTTCTAACTCATACCCTTGG-3'	<i>cpe</i>	166	
PrMY23	5'-CTCCATCACCTAAGGACTGTTC-3'	<i>cpe</i>		
PrMY217	5'-GTGCTACAATAGTTGGGATGGA-3'	<i>codY</i>	94	88
PrMY218	5'-CCAATAGCTAATTGAACCACTGC-3'	<i>codY</i>		
PrMY219	5'-CGCAGAAAAGGATGCTTTAGA-3'	<i>abrB</i>	100	
PrMY220	5'-AACATCTCTAGCATCTCCGCA-3'	<i>abrB</i>		
PrMY227	5'-AAATATTCTGGAGGAGCACA-3'	<i>agrB</i>	371	89
PrMY222	5'-CATTGCCATGCTACTCCAAA-3'	<i>agrB</i>		
PrMY223	5'-CCACAAGGAGAATATGAGGCTT-3'	<i>spoIIR</i>	125	
PrMY224	5'-TCATCATAGGCAACTTGTCCC-3'	<i>spoIIR</i>		
PrMY231	5'-TGCAACTGCATTGCCTGTA-3'	<i>spoIIGA</i>	179	
PrMY232	5'-CTCTTGTCATTGTTTCCCA-3'	<i>spoIIGA</i>		
PrMY44	5'-CGCATAATGTTGAAAGATGG-3'	16S rRNA	105	90
PrMY45	5'-CCTTGGTAGGCCGTTACC-3'	16S rRNA		

(T_m), the distance from the 3' terminus, %GC, score for probe quality on the base composition, and possible cross-hybridization against other positions in NCTC8239 and the genome of *Homo sapiens* (see Tables S1 and S2). Since the whole-genome sequence has not been completed for NCTC8239, we also determined whether the probes hybridized more than two positions in SM101 and *C. perfringens* ATCC 13124, the entire genome of which has been sequenced. Nucleotide homologies within the core genomic regions (the portion of the genome conserved among all taxa) for NCTC8239 were 99.0% and 97.8% in ATCC 13124 and SM101, respectively (42). The score for probe quality was divided into six grades (1 to 5 and poor). A lower score indicated that the probe had better quality. Every probe acquired had a score of 1 or 2. Therefore, we considered all probes to have sufficient qualities for use in microarrays on the base composition (see Table S1). In contrast, since 17 probes were likely to cross-hybridize with an unintended gene (see Table S2), we removed these probes for a microarray analysis. Therefore, a custom 8 × 15,000 oligonucleotide array containing 60-mer oligonucleotide probes for 2,761 genes in NCTC8239, two bacterial control genes (16S rRNA and 23S rRNA), and three human control genes (beta-2-microglobulin, glucuronidase beta, and 18S rRNA) was ordered from Agilent Technologies (design identifier 045370). Each probe was spotted 5-fold on each microarray.

Microarray. Gene expression analyses using an Agilent-045370 *C. perfringens* strain NCTC8239 8 × 15,000 array (Agilent), listed with the corresponding GEO accession number (GPL20295), were performed as one-color experiments. In order to efficiently remove most genomic DNA contamination without a DNase treatment, extracted total RNA was purified with an RNeasy MinElute cleanup kit (Qiagen) according to the manufacturer's instructions. The integrity of total RNA was confirmed using the RNA 6000 Nano LabChip kit on an Agilent 2100 Bioanalyzer (Agilent), and only samples with an RNA integrity number (RIN) greater than 6.0 were used in the microarray analysis. Total RNA (100 ng) was reverse transcribed into double-stranded cDNA with oligo(dT) and random primers using AffinityScript multiple-temperature reverse transcrip-

tase (Agilent). The resulting cDNAs were labeled with cyanine-3-labeled cytosine triphosphate (PerkinElmer, Waltham, MA) using an Agilent low-input quick amp WT labeling kit (Agilent). After the labeled cRNAs had been fragmented, each cRNA sample (600 ng) was hybridized on an Agilent 045370 *C. perfringens* strain NCTC8239 8 × 15,000 array (Agilent) at 65°C for 17 h with rotation in the dark. Hybridization was performed using a gene expression hybridization kit (Agilent) by following the manufacturer's instructions. After washing, the slides were scanned with an Agilent microarray scanner (G2505C). Feature extraction software (version 10.5.1.1) was used to convert images into gene expression data. In order to filter out spots lower than the background intensity, the probes with Agilent background-subtracted signals (gIsWellAboveBG = 1) were used in an expression analysis. Microarray data were normalized with the bacterial control genes and log₂ transformed, and the between-sample fold change then was calculated using the one-sample *t* test with Subio platform v1.15 (Subio). Each gene was annotated based on the NCBI (http://www.ncbi.nlm.nih.gov/genome/proteins/158?genome_assembly_id=159657) and PATRIC (<https://www.patricbrc.org/portal/portal/patric/Home>) databases.

qRT-PCR. Total RNA was isolated from the biological samples that were separate from those used in the microarray analyses. Total RNA was treated with DNase I (RQ1 RNase-free DNase; Promega, Madison, WI) according to the manufacturer's instructions. cDNA was synthesized using Superscript III reverse transcriptase (Life Science Technologies) with random primers (Life Science Technologies). Synthesized cDNA was subjected to a SYBR green real-time PCR assay (Life Science Technologies) with gene-specific forward and reverse primer sets (Table 1) using the Applied Biosystems StepOne real-time PCR system. Quantitative reverse transcription-PCR (qRT-PCR) data were normalized using 16S rRNA values. All reactions were performed in triplicate.

Mutant strain construction. The Spo0A phosphorylation site mutant (strain MY97) was prepared by complementing an *spo0A* null mutant with an Spo0A expression plasmid encoding a D58A (replacing Asp with Ala at position 58) nonphosphorylatable mutant. The phosphorylation

site of Spo0A was predicted by the conserved protein domain family on the NCBI database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=238088>) and located at Asp58 of the REC domain in SM101. The promoter and open reading frame (ORF) region of *spo0A* were PCR amplified from the genomic DNA of SM101 as described previously (39) and inserted into the pGEM-T Easy vector (Promega). Using this plasmid, the mutant *spo0A* gene encoding the mutated sequence of the phosphorylation site replacing Asp (D) with Ala (A) at amino acid position 58 was generated by site-directed mutagenesis PCR (GeneTailor site-directed mutagenesis system; Life Science Technologies) using the primer set PrMY145 and PrMY147 (Table 1). After we confirmed that the sequence was mutated correctly at amino acid position 58 by a Sanger sequencing analysis (Macrogen Japan, Tokyo, Japan), the promoter and mutated ORF region of *spo0A* were subcloned into the expression vector pJIR751 (43) to create pMY47. In order to construct the mutant strain MY97, pMY47 was introduced into strain IH101, the *spo0A* null mutant of SM101 (39), through electroporation. Transformants were selected on BHI agar containing 30 $\mu\text{g/ml}$ of erythromycin. Thus, the mutant we prepared expressed the mutated Spo0A *in trans* by transforming the mutated expression plasmid into the *spo0A*-null mutant.

Detection of Spo0A phosphorylation using a Phos-tag acrylamide gel. A Phos-tag acrylamide gel analysis was performed as described previously (44), but with slight modifications. Phos-tag acrylamide gels were prepared according to the instructions provided (Wako); 10% gels were copolymerized with 25 μM Phos-tag acrylamide and 50 μM MnCl_2 . The 24-ml bacterial cultures (optical density at 600 nm [OD_{600}] of 0.4 to 0.5) collected were centrifuged at $5,000 \times g$ at 4°C for 10 min, and the pellets were lysed with 300 μl of 1 M formic acid. They were transferred into a tube containing zirconia beads, vortexed for 4 min for complete lysis of the pellets, and centrifuged at $21,130 \times g$ at 4°C for 3 min. The supernatants obtained were solubilized by the addition of SDS loading buffer containing β -mercaptoethanol and subsequently neutralized by the addition of 5N NaOH. Samples were stored on ice prior to loading onto Phos-tag acrylamide gels and run at 4°C . Gels were fixed for 10 min in transfer buffer with 10 mM EDTA and then washed for 10 min in transfer buffer without EDTA twice. After being transferred to a polyvinylidene difluoride (PVDF) membrane, the samples were probed with a rabbit polyclonal anti-Spo0A antibody (45) at 37°C for 1 h. Horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) then was reacted at 37°C for 1 h and detected with ECL prime Western blotting detection reagent (GE Healthcare, Little Chalfont, United Kingdom). In order to dephosphorylate Spo0A~P, samples were heated at 100°C for 5 min.

Statistical analyses. Data are expressed as means \pm standard deviations (SD) or standard errors of the means (SEM). Statistical analyses were performed using the Student *t* test or Mann-Whitney U test. $P < 0.05$ was considered significant. Statistical analyses for microarrays performed were as described above in the "Microarray" section.

Microarray data accession numbers. The microarray data used in this study have been deposited in the NCBI Gene Expression Omnibus database (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE69649 (see also GPL20295).

RESULTS

DCA-induced sporulation in *C. perfringens* strain NCTC8239.

In order to gain insights into the mechanism underlying DCA-induced sporulation in a *C. perfringens* FBI strain (25), we cocultured NCTC8239 with Caco-2 cells in DMEM(–) containing starch alone or starch with DCA [S/DMEM(–) or S/D/DMEM(–), respectively] in a CO_2 incubator and examined the kinetics of bacterial growth and sporulation (Fig. 1). Bacterial growth was not observed in the samples culturing bacterial cells in a 24-well plate in the absence of Caco-2 cells (25) or starch (data not shown). In the absence of DCA, heat-resistant spores appeared at 8 h postinoculation (hpi), and the number of spores

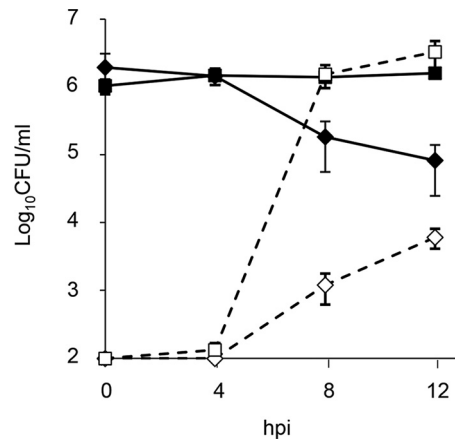


FIG 1 Bacterial growth and sporulation kinetics of NCTC8239 in response to DCA. NCTC8239 was cocultured with Caco-2 cells in S/DMEM(–) (diamonds) or S/D/DMEM(–) (squares) in a CO_2 incubator. The number of vegetative cells (solid lines) or spores (dashed lines) was determined at 0, 4, 8, and 12 hpi by plating serially diluted unheated (for vegetative cells) or heat-treated (for heat-resistant spores) samples, respectively, on BHI agar, incubating at 37°C for 24 h under anaerobic conditions, and counting CFU. Data represent the means \pm SEM from three independent experiments.

reached 5.0×10^3 CFU/ml by 12 hpi. The number of vegetative cells was sustained at 1.0×10^6 CFU/ml until 4 hpi but then decreased to 1.0×10^5 CFU/ml by 12 hpi. In contrast, the number of spores markedly increased (up to 1.0×10^6 CFU/ml) at 8 hpi in the presence of DCA. The number of vegetative cells was sustained at 1.0×10^6 CFU/ml during the experiment. These results were consistent with our previous findings (25) showing that the numbers of vegetative cells and spores both reached 1.0×10^6 CFU/ml at 8 hpi and were sustained until 24 hpi in S/D/DMEM(–), whereas the numbers of vegetative cells and spores in S/DMEM(–) reached 1.0×10^5 and 1.0×10^3 CFU/ml, respectively, at 8 hpi; both reached 1.0×10^4 CFU/ml at 24 hpi. SM101, a derivative of FBI strain NCTC8798, also showed that sporulation was enhanced in the presence of DCA; however, the effects of DCA were not as prominent as those in NCTC8239 (see Fig. S2 in the supplemental material). Therefore, we selected NCTC8239 for further study. These results indicated that DCA enhanced sporulation in our coculture system.

In order to determine which sporulation stage was enhanced by DCA, we analyzed bacterial morphology using fluorescence microscopy. NCTC8239 was cultured in DS medium for 2 to 8 h and stained by rhodamine B and Hoechst 33342. The stained cells were observed under the phase-contrast and fluorescence microscope and divided into eight morphological stages, as shown in Fig. 2A. During bacterial growth, we observed bacterial cells with or without symmetric membrane septation and the division of nuclei (cell division or vegetative cells, respectively). Cells with an asymmetric septum or engulfed membrane during the initial phase of sporulation were defined as stage II. Stage III featured the complete engulfment of the membrane and positive nuclear staining in prespores. Stages IV and V characterized cells containing gray endospores under the phase-contrast microscope with negative nuclear staining in prespores. Bright endospores were observed under the phase-contrast microscope at stage VI. Stage VII featured the lysis of the mother cell and positive nuclear staining around mature spores. Bright spores under the phase-contrast

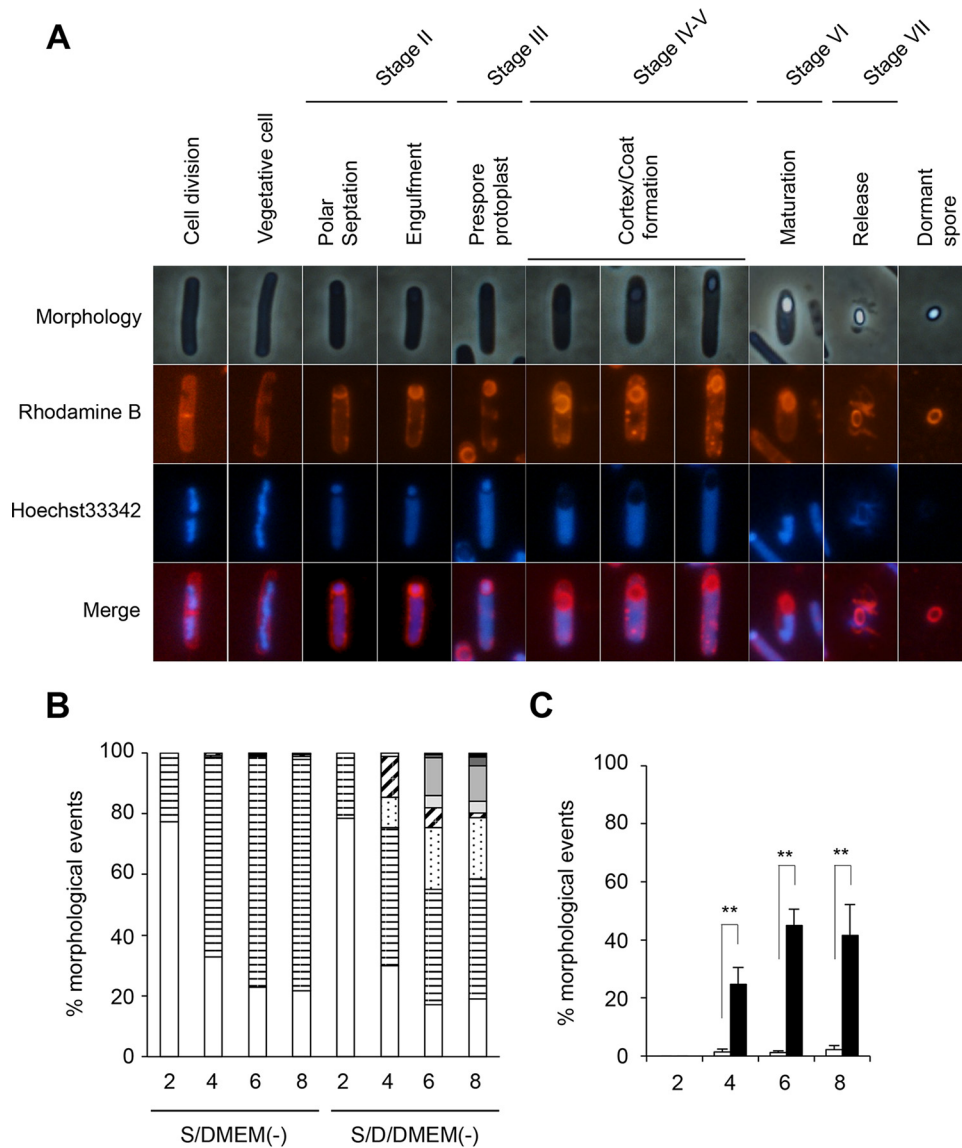


FIG 2 Morphological observations of sporulation in NCTC8239 in response to DCA. (A) NCTC8239 cultured in DS medium was stained by rhodamine B and Hoechst 33342 for the cell membrane and double-stranded DNA, respectively. Samples were observed under a phase-contrast microscope with fluorescence accessories. The panels of each stage correspond to the stages defined in the sporulation of *B. subtilis* (87). (B) NCTC8239 was cocultured with Caco-2 cells in S/DMEM(–) or S/D/DMEM(–) and stained with rhodamine B and Hoechst 33342 at 2, 4, 6, and 8 hpi. The stained cells were divided into eight morphological stages, as shown in panel A, and counted. The total number of cells counted was more than 1,000 in each sample. Data represent the means from three independent experiments. Cell division, white; vegetative cell or stage 0, stripe; stage II, dot; stage III, oblique; stages IV to V, light gray; stage VI, medium gray; stage VII, dark gray; dormant spore, black. (C) The bacterial population in the sporulation process (stages II, III, IV–V, VI, and VII and dormant spores) was calculated in samples identical to those used for panel B in S/DMEM(–) (open bars) and S/D/DMEM(–) (solid bars). All data represent the means \pm SD from three independent experiments. **, $P < 0.01$ [significantly different from results for S/DMEM(–)].

microscope were defined as dormant spores. When bacterial cells were cocultured with Caco-2 cells in S/DMEM(–) for 2, 4, 6, and 8 h, most of the bacterial population was in the growing cycle (cell division and vegetative cells) (Fig. 2B). In contrast, more than 40% of the bacterial population cocultured in S/D/DMEM(–) switched to the sporulation process (stages II, III, IV–V, VI, and VII and dormant spores) (Fig. 2B). The bacterial population in the sporulation process was significantly larger in S/D/DMEM(–) than in S/DMEM(–) (Fig. 2C). These results indicate that DCA stimulates the early events of sporulation, such as the activation of the master regulator Spo0A.

Transcriptome analysis of NCTC8239 during DCA-induced sporulation. In order to understand the comprehensive gene expression pattern in NCTC8239 in response to DCA, we compared gene expression profiles in *C. perfringens* cells cocultured with Caco-2 cells in the presence or absence of DCA at 1, 2, 3, 4, 6, and 12 hpi using DNA microarrays. The whole-genome sequence has not yet been completed in NCTC8239, whereas SM101 has already had its entire genome sequenced (46). Nevertheless, the genome length and number of genes and proteins were larger in NCTC8239 (3.32 kb; 3,068 genes and 2,784 proteins) than in SM101 (2.90 kb; 2,765 genes and 2,619 proteins) (42). The 2,761

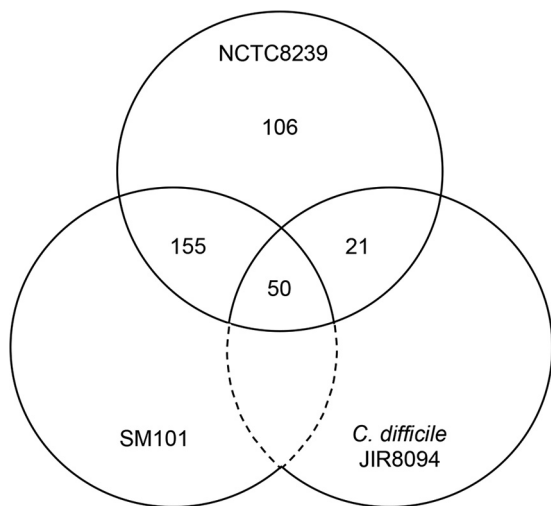


FIG 3 Venn diagrams indicating numbers of regulated genes identified in NCTC8239, SM101, and/or *C. difficile*. NCTC8239 was cocultured with Caco-2 cells in S/DMEM(–) or S/D/DMEM(–) for 1 to 12 h, and total RNA extracted was subjected to a DNA microarray analysis. The 332 genes significantly upregulated during DCA-induced sporulation in NCTC8239 were compared to the orthologs identified as being upregulated during sporulation in SM101 (32) and as being regulated by Spo0A/sigma factors in *C. difficile* JIR8094 (29).

coding sequences for the microarray analysis in this study covered most of the genes and proteins identified (90.00% and 99.17%, respectively). Therefore, we considered it possible to characterize most genes involving DCA-induced sporulation in NCTC8239. Microarray experiments were performed three times for each time point, and triplicate data sets were statistically analyzed (Fig. S3; also see Table S3 in the supplemental material). Genes showing at least 4-fold differential expression levels from those in samples for comparison and with a confidence level (*P* value) of less than 0.05 were selected and defined as differentially expressed genes. A total of 333 genes (12.06% of the genes analyzed) were differentially expressed in response to the addition of DCA in at least one time point during the experiment (see Table S4). No gene was differentially expressed at 1 or 2 hpi, whereas the expression of 7, 162, 315, and 101 genes was changed at 3, 4, 6, and 12 hpi, respectively. Of these, only one gene [AC7_0824; encoding lipoprotein, iron(III) dicitrate transport system, and periplasmic iron-binding protein FecB] was downregulated at 4 hpi, whereas all other genes were upregulated in response to the addition of DCA.

In order to identify the genes specifically induced in the presence of DCA in NCTC8239, we compared the 333 genes differentially expressed in our study with those previously elucidated as being regulated during sporulation in SM101 (32) and with Spo0A/sigma factor-regulated genes reported in *C. difficile* strain JIR8094 (29). We first determined the orthologs of our 333 genes with SM101 and *C. difficile* using blastn or blastp of a BLAST search on the NCBI database, respectively, with an E value cutoff of 0.0001 (47) (see Table S4 in the supplemental material). The ortholog of AC7_0824, which was downregulated during DCA-induced sporulation, was positively regulated during sporulation in SM101 (see Table S4). Of the 332 genes upregulated during DCA-induced sporulation, 50 genes were sporulation related in SM101 and also Spo0A/sigma factor regulated in *C. difficile* (Fig. 3;

also see Tables S5 and S6). A total of 155 genes were upregulated during SM101 sporulation (Fig. 3; also see Table S5). Twenty-one genes were Spo0A- or sigma factor-regulated genes in *C. difficile* but were not upregulated during sporulation in SM101 (Fig. 3; also see Table S6). Therefore, we found that DCA led to the upregulation of 106 genes that appear to be unrelated to the process of sporulation based on comparisons with previously published SM101 and *C. difficile* sporulation transcriptomes (Fig. 3; also see Table S7).

Thirty-three out of the 106 genes were defined as a putative protein, hypothetical protein, or conserved domain protein. Therefore, we focused on the remaining 73 genes and applied the Cluster of Orthologous Groups of proteins (COG) classification (48) in order to further characterize the genes in NCTC8239. We conducted a BLAST search for each gene in NCTC8239 with *C. perfringens* strain 13 and/or SM101, in which the genes had been classified by COG, and then sorted the genes functionally (Fig. 4; also see Table S7 in the supplemental material). In the group of energy production and conversion (group C), *glpK* was predicted to comprise an operon with the operon antiterminator gene *glpP* belonging to group K, which also was specifically stimulated by DCA. These are known to be under the control of carbon (glycerol and glucose) catabolite repression in *Bacillus subtilis* (49). *mbl* in the group of cell cycle control and division (group D) was predicted to be located in the same operon as *spoIIID*, encoding the sporulation-related protein SpoIIID. In the group of amino acid transport and metabolism (group E), *arcA*, *argF*, *arcD*, and *arcC* were predicted to comprise an operon. *pepT*, encoding a hydrolase, was involved in germination in *Bacillus anthracis* (50). As for AC7_0201, encoding sodium/solute symporter family proteins, a bacterial homolog of the apical sodium-dependent bile acid transporter was detected in *Neisseria meningitidis* (51), implying that deoxycholate can be absorbed in the cytoplasm in NCTC8239 through this symporter. Gcn5-related N-acetyltransferase is required for the recovery of non-N-acetylated muropeptides during cell wall turnover in *Clostridium acetobutylicum* (52). *dapA* and *dapB* encode the precursor synthetic enzymes for dipicolinic acid and were activated during sporulation in *Bacillus cereus* (53, 54). As for AC7_2228, encoding nucleoside diphosphate kinase in the group of nucleotide transport and metabolism (group F), the phosphodonor Spo0B of *B. anthracis* exhibited the diphosphate kinase-like activity of phosphate transfer from nucleoside triphosphate to nucleoside diphosphate (55). Furthermore, the nucleoside diphosphate kinase of *P. aeruginosa* showed 77% homology at the N terminus with Spo0E, a negative regulator of sporulation in *B. subtilis* (56). In the group of carbohydrate transport and metabolism (group G), AC7_0583, encoding the ABC transporter, was predicted to comprise an operon with a two-component system of AC7_0581 (sensor histidine kinase) and AC7_0582 (AraC family DNA-binding response regulator). AC7_0581 also was stimulated specifically in response to DCA (group T), and the ortholog of AC7_0582 was a Spo0A-dependent gene in *C. difficile* (29). The orthologs of *glgC*, AC7_A0087 (encoding glycogen synthase) and *glgB*, are known as the σ^E regulon in *B. subtilis* (57). In the group of lipid transport and metabolism (group I), *nagH*, encoding hyaluronidase (μ -toxin), was shown to be regulated by the DNA-responding regulator RevR in *C. perfringens* strain 13 (58, 59). In the group of translation (group J), *argS*, *cysS*, and *ileS*, encoding arginyl-, cysteinyl-, and isoleucyl-tRNA synthetase, respectively, were specifically upregulated for DCA. It has not yet

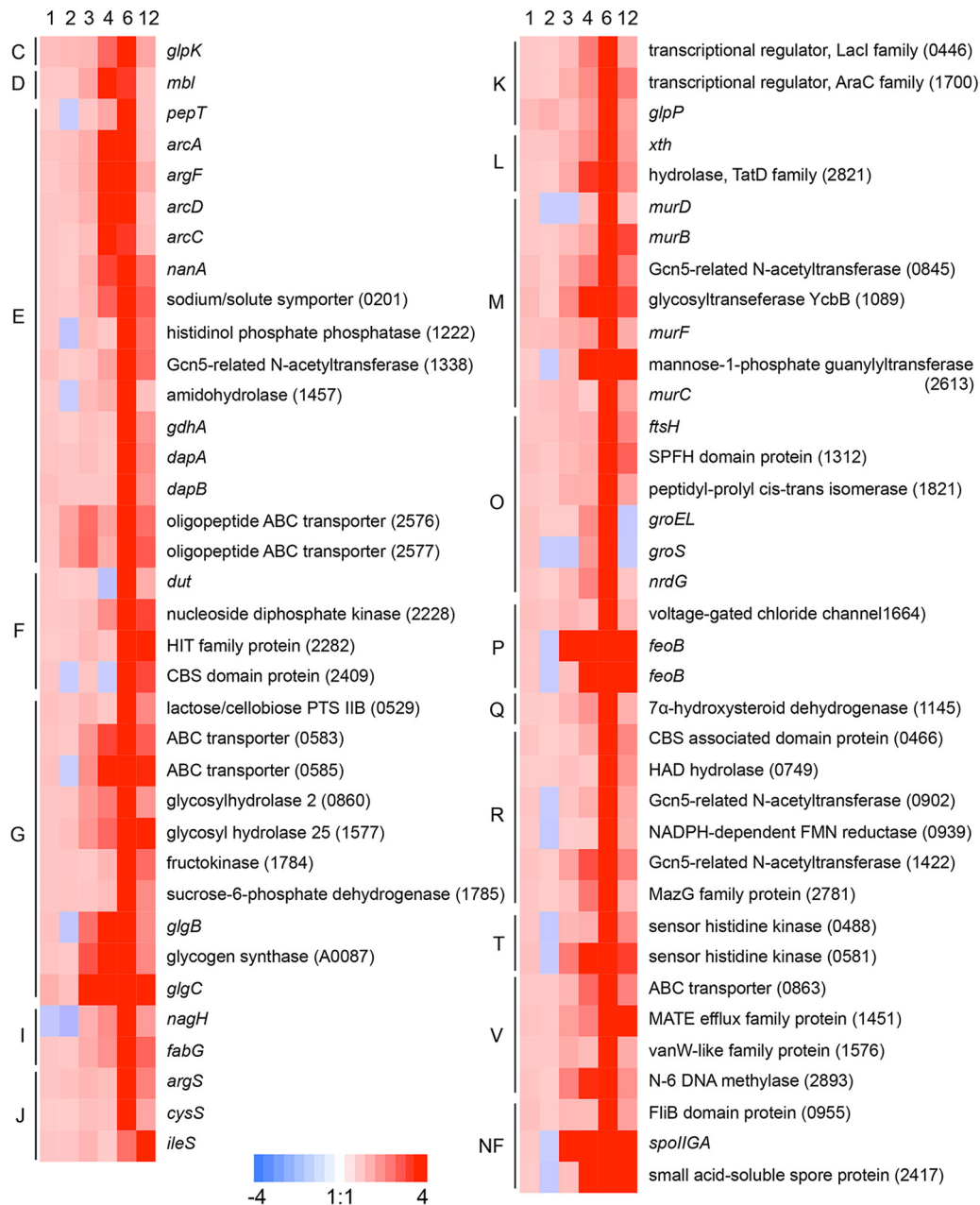


FIG 4 Differentially expressed genes specific for DCA-induced sporulation in NCTC8239. Of the 106 genes specifically induced by DCA as shown in Fig. 3, 73 genes with known functions were divided based on the COG classification. The heat maps indicate the ratio of gene expression levels in S/D/DMEM(–) to those in S/DMEM(–) at identical time points (1 to 12 h). The gene or product names are shown on the right of the maps. The numbers shown in parentheses indicate locus tags. COG classifications are on the left sides of the maps: C, energy production and conversion; D, cell cycle control, cell division, and chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure, and biogenesis; K, transcription; L, replication, recombination, and repair; M, cell wall/membrane/envelope biogenesis; O, posttranslational modification, protein turnover, and chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolite biosynthesis, transport, and catabolism; R, general function prediction only; T, signal transduction mechanisms; V, defense mechanisms; NF, no COG found. All data represent the means from three independent experiments. Details of differentially expressed genes are shown in Table S7 in the supplemental material.

been established whether they are important for DCA-induced sporulation, while valyl-tRNA synthetase altered its activity during the early stage of sporulation and was closely related to the sporulation of *B. subtilis* (60), suggesting that they are essential genes for DCA-induced sporulation in NCTC8239. In the group of transcription (group K), AC7_0446, encoding the LacI family

transcriptional regulator, has been identified as a negative regulator of the maltose operon (61, 62). In the group of cell wall/membrane biogenesis (group M), the orthologs of *murB*, *murC*, and *murF* belong to the σ^K regulon in *B. subtilis* (63). In the group of posttranslational modifications (group O), the ortholog of *ftsH* is known as a general stress gene and was shown to be involved in

sporulation in *B. subtilis* (64). The upregulation of *groEL* and *groS* was consistent with previous findings showing that bile salts induce chaperone proteins to repair misfolded proteins in *B. cereus* (65). The expression of *nrdG* encoding the anaerobic ribonucleoside-triphosphate reductase-activating protein was upregulated in response to DCA, whereas the target gene encoding anaerobic ribonucleoside-triphosphate reductase (AC7_0944) was not differentially expressed in the presence of DCA (see Table S3). The expression of iron storage proteins was modulated during bile exposure in *Helicobacter pylori* (66), and iron has been shown to induce sporulation in *Bacillus* spp. (67); however, it is not yet clear whether DCA-induced *feoB*, encoding the iron transporter and belonging to the group of inorganic ion transport and metabolism (group P), is important for sporulation. AC7_1145, encoding 7- α -hydroxysteroid dehydrogenase in the group of secondary metabolite biosynthesis and catabolism (group Q), is known as an enzyme for bile acid transformation produced by intestinal microbiota (68). In the group of signal transduction mechanisms (group T), the expression of AC7_0488, encoding the sensor histidine kinase, was upregulated, while that of its response regulator (AC7_0487) was not altered during DCA-induced sporulation in NCTC8239 (see Table S3). In the group undifferentiated by COGs containing 3 genes, *spoIIGA* and AC7_2417, encoding a small acid-soluble spore protein, are defined as sporulation-related genes.

In summary, various functional types of genes were differentially expressed during DCA-induced sporulation in NCTC8239. Two-thirds of these genes were consistent with previous findings obtained with sporulation-related genes in SM101 and/or *C. difficile*. In the remaining one-third, some genes were reported to be involved in bile stress or the sporulation process in *Clostridium* spp., *Bacillus* spp., and/or other bacteria, while the mechanisms by which the other genes are involved in DCA-induced sporulation have not yet been elucidated. They may be essential for inducing sporulation by DCA.

Sporulation-related gene expression of NCTC8239 during DCA-induced sporulation. Of the 2,761 genes analyzed, we extracted 78 sporulation-related genes based on the NCBI and PATRIC databases (see Table S8 in the supplemental material). We divided them into two groups. One included the genes differentially expressed (upregulated) in response to the addition of DCA in at least one time point during the experiment (Fig. 5A and B), and the other included genes whose expression was not significantly altered during the experiment (Fig. 5C and D). We then compared each of them with the orthologs in the previous findings obtained with SM101 (32) and *C. difficile* (29) in order to elucidate whether gene expression is specific for DCA-induced sporulation in NCTC8239. Forty-eight out of the 50 upregulated genes also were stimulated during sporulation in SM101 and/or regulated by Spo0A or sigma factors in *C. difficile* (Fig. 5A). *spoIIGA* and AC7_2417, encoding a small acid-soluble protein, were specifically stimulated during DCA-induced sporulation in NCTC8239. Eighteen out of the 28 genes not differentially expressed also were not stimulated in SM101 or *C. difficile* (Fig. 5C). The expression of the remaining 10 genes, including *spoIID*, *spoIVB*, AC7_2278, *spoVAC*, *spoVT*, *cotJC*, *gpr*, *ylbJ*, AC7_2160, and *yvtI*, was not altered specifically in DCA-induced sporulation in NCTC8239.

We next divided 78 genes into the sporulation stages in order to elucidate which sporulation stage is activated by DCA (Fig. 5B and D; also see Table S8 in the supplemental material). NCTC8239 had three or four homologs in *spoVB*, *spoVD*, and *cotS*. Of these,

each homolog in *spoVB* and *spoVD* showed different expression profiles during DCA-induced sporulation; some were dependent on sporulation, whereas others were not. It is important to note that no significant differences were observed in the expression patterns of *spo0A* in the presence or absence of DCA at any time point (Fig. 5D, stage 0). Furthermore, no significant differences were observed in the expression of genes in stage 0 in the presence or absence of DCA. In contrast, the expression of genes in stage II, including *spoIIAA* and *spoIIAB*, was markedly higher in S/D/DMEM(-) than in S/DMEM(-) at 3 or 4 hpi. Spo0A-regulated sigma factors, including *sigF* (predicted to comprise an operon with *spoIIAA* and *spoIIAB*), *sigE* (predicted to comprise an operon with *spoIIGA* and *sigG*), *sigG*, and *sigK* also were strongly expressed in the presence of DCA at 4 hpi. The expression of a large number of genes in stages III and IV was significantly stronger in cultures with DCA at 4 hpi. The expression of many stage V genes, spore coat genes, and other sporulation-related genes also was significantly upregulated at 4 or 6 hpi. These results were consistent with DCA increasing the cell population of the sporulation process, as shown in Fig. 2C.

We then performed qRT-PCR using newly prepared samples in order to confirm the microarray results (see Fig. S4 in the supplemental material). The samples were not amplified without reverse transcription. Consistent with the microarray data, DCA significantly upregulated the expression of *spoIIAA*, *spoIIAB*, *sigF*, *spoIIGA*, and *sigE*, whereas that of *spo0A* and *spoIIR* was not altered in the presence or absence of DCA at 4 hpi. Taken together, these results demonstrated that DCA activated Spo0A-regulated genes but not *spo0A* itself during sporulation.

Detection of Spo0A~P. In *Bacillus* spp., the Spo0A protein is activated through its phosphorylation by histidine kinase(s) followed by dimerization (69). The activated Spo0A protein binds to DNA promoter regions containing the Spo0A-binding motif, called the 0A box (TGNCGAA), and then regulates the expression of Spo0A-regulated genes (70). Therefore, we hypothesized that DCA activates the Spo0A protein. In order to compare the amount of Spo0A~P in the presence or absence of DCA, NCTC8239 was cocultured with Caco-2 cells in S/DMEM(-) or S/D/DMEM(-) for 4 h, and cell lysates were loaded onto Phos-tag acrylamide gels (Fig. 6A). We detected two bands, one as a slowly migrating band and another at a lower position. The amount of the upper band was greater in the presence of DCA (Fig. 6A, lane 3) than in its absence (Fig. 6A, lane 2). In the sample cultured in DS sporulation medium in SM101 (Fig. 6A, lane 4), we found results similar to those observed in NCTC8239. We then constructed an SM101 strain harboring the missense gene mutation (D58A) for the phosphorylation site of Spo0A. No shifted bands were detected in the samples of the mutant strain (MY97) cultured in DS sporulation medium (Fig. 6A, lane 5). We also found that the upper bands disappeared when the samples were heated. Taken together, these results indicate that the upper band is phosphorylated Spo0A and the lower band is the unphosphorylated form, and also that the amount of Spo0A~P is higher in S/D/DMEM(-) than in S/DMEM(-). We cocultured bacterial cells with Caco-2 cells in DMEM(-) containing glucose [G/DMEM(-)], because glucose is known to inhibit sporulation by regulating the transcription of *spo0A* (71). Bacterial cells did not sporulate in G/DMEM(-) until 24 hpi (25) even with the addition of DCA (data not shown). We found that the upper band was not detected in G/DMEM(-) in Phos-tag analyses (Fig. 6A, lane 1), suggesting

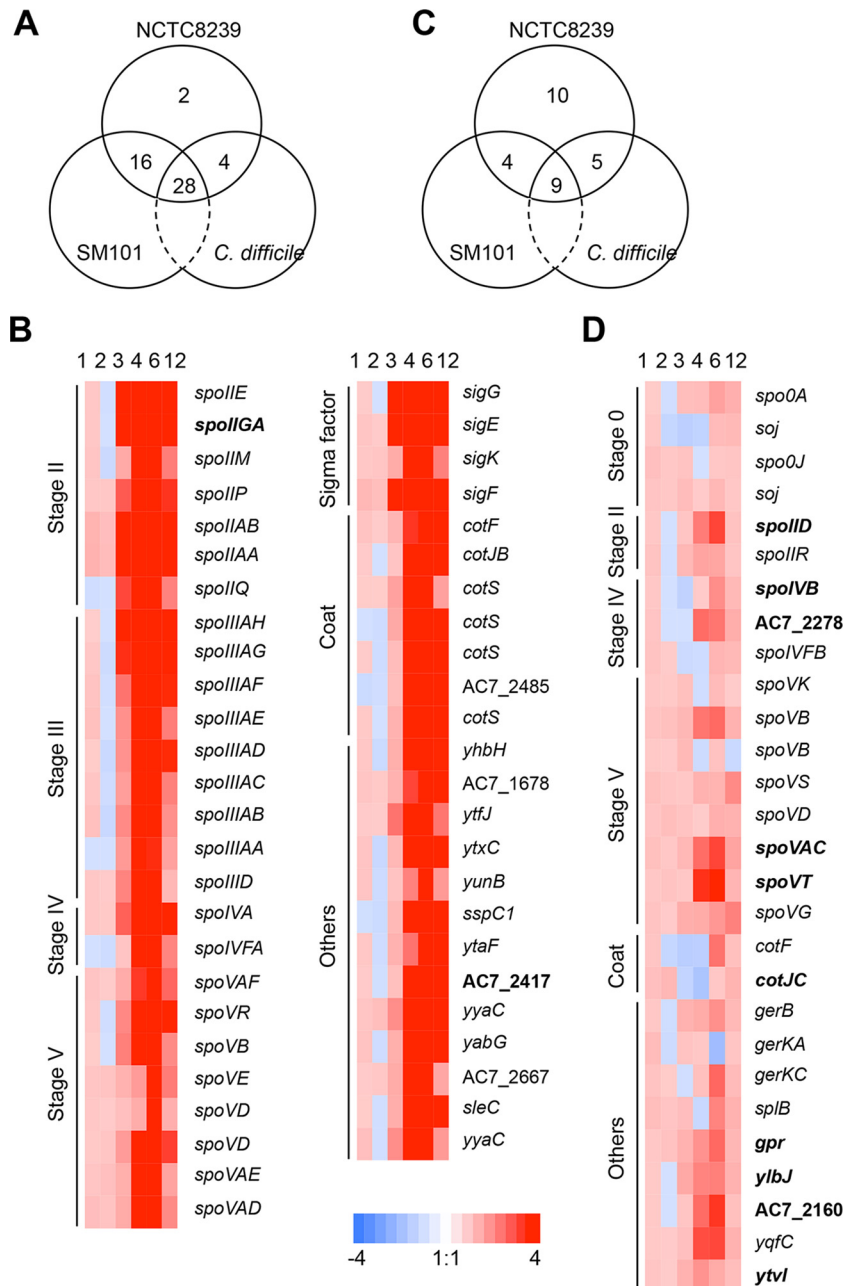


FIG 5 Transcriptional analysis of genes related to sporulation. Based on microarray data, 78 sporulation-related genes were extracted and divided into two groups: one was significantly upregulated during at least one time point (A and B), and the expression of the other was not altered during the experiment (C and D). (A and C) Venn diagrams of genes upregulated (A) or not altered (C) in this study and the orthologs previously reported in SM101 (32) and *C. difficile* (29) as shown in Fig. 3. (B and D) The heat maps indicate the ratio of gene expression levels of genes upregulated (B) or not altered (D) in S/D/DMEM(-) to those in S/DMEM(-) at identical time points (1 to 12 h). The gene names and stages in the sporulation process are shown on the right and left sides of the heat maps, respectively. The genes in boldface indicate that their expression was specific for DCA-induced sporulation in NCTC8239, as shown in panels A and C. All data represent the means from three independent experiments. The details of gene expression are shown in Table S8 in the supplemental material.

that glucose would inhibit the phosphorylation of Spo0A in addition to *spo0A* transcription. We then quantified band intensities in the absence and presence of DCA in order to estimate the ratio of the phosphorylated to unphosphorylated form of Spo0A using Image-J software. The ratio of Spo0A~P to the unphosphorylated form of Spo0A was 6.4-fold higher in S/D/DMEM(-) than in S/DMEM(-) (Fig. 6B). These results indicated that the phos-

phorylation of the Spo0A protein was facilitated in the presence of DCA.

Search for candidate genes increasing Spo0A~P. In *Bacillus* spp., three histidine kinases are mainly involved in Spo0A phosphorylation (69, 70), whereas the genes corresponding to the kinases are not conserved in clostridia. In some *Clostridium* spp., orphan histidine kinases have been identified as direct activators

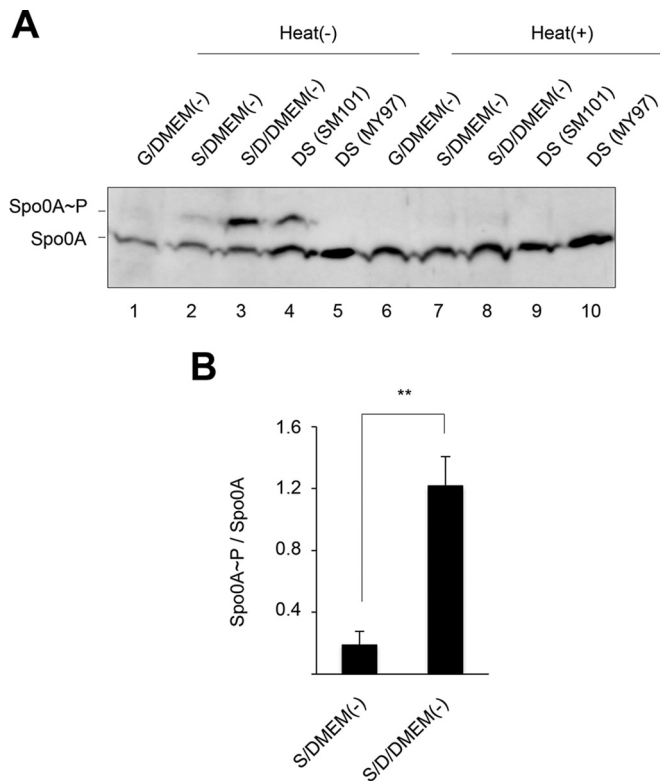


FIG 6 Detection of Spo0A phosphorylation. (A) NCTC8239 was cocultured with Caco-2 cells in S/DMEM(-) (lane 2), S/D/DMEM(-) (lane 3), or DMEM(-) containing glucose [G/DMEM(-)] (lane 1) for 4 h. The cell lysates collected were loaded onto a Phos-tag acrylamide gel and subjected to Western blot analysis using an Spo0A-specific antibody. Lower bands are the unphosphorylated form of Spo0A (Spo0A), and upper bands indicate the phosphorylated form of Spo0A (Spo0A~P). DS(SM101) or DS(MY97), the lysate of strain SM101 (expressing wild-type Spo0A) (lane 4) or MY97 (expressing non-phosphorylatable Spo0A) (lane 5) cultured in DS broth for 4 h, was used as a positive or negative control, respectively, for the phosphorylation of Spo0A. Samples identical to those of lanes 1 to 5 were heated at 100°C for 5 min and loaded as the total amount of unphosphorylated Spo0A (lanes 6 to 10). (B) The ratio of Spo0A~P to Spo0A in S/DMEM(-) (lane 2) and S/D/DMEM(-) (lane 3) was assessed with Image J software. All data represent the means \pm SD from three independent experiments. **, $P < 0.01$ [significantly different from S/DMEM(-)].

of Spo0A (34–36). In order to determine which orphan kinases are essential for Spo0A phosphorylation, we identified 8 orphan histidine kinases from 2,778 coding sequences in NCTC8239 based on blastp analyses with kinases from *C. perfringens* strains 13 and SM101 (35). We then examined their expression profiles in our microarray data sets because the expression of genes encoding histidine kinases was induced at the beginning of sporulation in *B. subtilis* (33, 72). However, none of the genes were differentially expressed (i.e., reached the threshold defined by us) in the presence of DCA in NCTC8239 (Table 2).

In an attempt to identify other candidate genes that increase Spo0A~P, we extended our focus to the genes upregulated at 3 hpi in the presence of DCA, because several Spo0A-regulated genes (*spoIIAA*, *spoIIAB*, *spoIIIE*, and *spoIIIGA*) already were induced at that time point (Fig. 5B; also see Table S8 in the supplemental material). We found that three genes (AC7_0229, A0092, and A0093) were significantly induced by DCA (see Tables S3 and S4). AC7_0229 encoded a hypothetical protein. Its orthologs also were found in *C. perfringens* strains 13, SM101, and ATCC 13124 from the blastp search; however, the gene did not appear to be conserved in other clostridia. Thus, although it is conceivable that AC7_0229 plays a role in DCA-induced Spo0A activation, the putative cascade must be specific to *C. perfringens* strains. Another candidate, AC7_A0092, encoded the glucose-1-phosphate adenylyl transferase GlgD subunit. This enzyme catalyzes the conversion of alpha-D-glucose 1-phosphate to ADP-glucose and participates in starch and sucrose metabolism (73). It currently remains unclear whether the carbohydrate-metabolizing enzyme is directly involved in the activation of Spo0A. The other candidate, AC7_A0093, encodes phosphotransbutyrylase, which catalyzes the conversion of butyryl-coenzyme A (CoA) to butyryl phosphate (BuP) (74). This gene possesses an Spo0A box in its promoter region (75). It is noteworthy that the high level of BuP induced by a defective mutant of butyrate kinase, which converts BuP to butyrate, accelerated sporulation in *C. acetobutylicum* (33, 74). Collectively, we found three upregulated genes that may contribute to the phosphorylation of Spo0A in DCA-induced sporulation.

Expression of genes regulating virulence. *C. perfringens* strains produce various extracellular enzymes and toxins through a global regulation system (76). For example, the production of α -toxin, θ -toxin, κ -toxin, proteases, and sialidases is known to be

TABLE 2 Expression of genes encoding orphan histidine kinases

Locus tag	Gene expression by time point ^a											
	1 h		2 h		3 h		4 h		6 h		12 h	
	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value
AC7_0230	1.08	0.86	-1.03	0.95	1.52	0.35	-1.11	0.69	1.57	0.41	-1.03	0.96
AC7_0895	-1.19	0.86	-1.01	0.99	-1.43	0.61	-1.58	0.61	1.31	0.61	1.33	0.70
AC7_1186	1.12	0.79	-1.03	0.95	1.15	0.72	1.13	0.28	2.51	0.03	1.62	0.25
AC7_1236	1.07	0.29	1.05	0.68	-1.12	0.35	-1.07	0.71	1.11	0.61	-1.00	0.98
AC7_1579	1.21	0.38	1.18	0.65	2.19	0.10	2.89	0.02	4.83	0.06	2.00	0.17
AC7_1765	1.11	0.76	1.13	0.80	1.22	0.58	-1.25	0.14	3.82	0.02	1.63	0.26
AC7_2012	1.04	0.98	-1.02	0.99	1.33	0.73	1.46	0.31	2.07	0.07	1.44	0.40
AC7_2242	1.25	0.33	1.11	0.76	1.50	0.23	1.64	0.01	2.68	0.10	1.67	0.26
AC7_2243	1.32	0.56	-1.09	0.75	-1.42	0.61	-1.40	0.63	1.79	0.25	1.52	0.37

^a Fold changes in gene expression in S/D/DMEM(-) relative to that in S/DMEM(-) are shown.

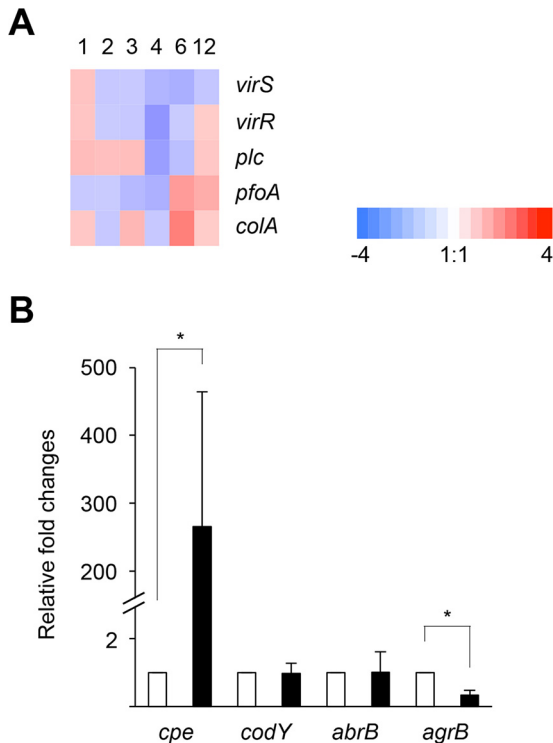


FIG 7 Expression of virulence-associated genes during DCA-induced sporulation. (A) The heat map indicates the ratio of gene expression levels in S/D/DMEM(-) to those in S/DMEM(-) at identical time points (1 to 12 h). All data represent the means from three independent experiments. The details of gene expression are shown in Table S3 in the supplemental material. (B) NCTC8239 was cocultured with Caco-2 cells in S/DMEM(-) (open bars) or S/D/DMEM(-) (solid bars). RNA was extracted from samples at 4 hpi and subjected to qRT-PCR for *cpe*, *codY*, *abrB*, and *agrB*. The y axis represents relative fold changes in the gene expression levels of S/D/DMEM(-) against S/DMEM(-). All data represent the means \pm SEM from three independent experiments. *, $P < 0.05$ [significantly different from S/DMEM(-)].

positively regulated by the two-component VirR/VirS system and *agr* system (76). *C. perfringens* strains also produce sporulation-dependent CPE. CodY has been shown to function as a global regulator of virulence-associated properties in the type D strain (77), and AbrB has been identified as a global repressor involved in sporulation and biofilm formation in *B. subtilis* (78) and a *C. perfringens* type A strain (79). Therefore, we determined whether DCA modified the expression of these virulence-associated genes. Microarray data revealed that there was no evidence to show that DCA induced the expression of *virR-virS* or VirRS-VR regulons such as *plc*, *pfoA*, and *colA*, encoding α -toxin, θ -toxin, and κ -toxin, respectively (Fig. 7A). The 2,778 coding sequences lacked several genes, including *cpe*, *codY*, *abrB*, and *agrB*. Therefore, we performed qRT-PCR at 4 hpi in order to investigate whether these genes were involved in DCA-induced sporulation (Fig. 7B). As expected, DCA significantly upregulated the expression of *cpe*, which is consistent with our previous findings showing DCA-induced CPE production (25). In contrast, the expression of *codY* and *abrB* was not altered in the presence or absence of DCA. The expression of *agrB* decreased significantly in the presence of DCA, suggesting that DCA regulates the *agr* system negatively. These results indicate that DCA induced sporulation-regulated toxin gene expression but did not stimulate a major toxin-regulating

system, such as the VirR/VirS system or the global transcriptional regulators *codY* and *abrB*.

DISCUSSION

Bile has been shown to induce sporulation (22–25); however, the mechanisms underlying bile-induced sporulation have not yet been elucidated in detail. To the best of our knowledge, we are the first to demonstrate that DCA induces sporulation, at least partially, by enhancing the phosphorylation of Spo0A and activating Spo0A-regulated genes. The increased expression of Spo0A-regulated genes in our study was attributed to the facilitation of Spo0A phosphorylation, because the Spo0A protein is activated through phosphorylation by histidine kinase(s) followed by dimerization and controls the expression of Spo0A-regulated genes (69, 70). Further studies are needed to determine whether DCA-induced sporulation is exclusively dependent on increases in the phosphorylation of Spo0A.

The mechanism by which DCA affects the phosphorylation of Spo0A also needs to be clarified. One possibility is that DCA as a ligand stimulates the genes encoding histidine kinases, which directly activate the Spo0A protein. The expression of genes encoding histidine kinases was previously reported to be induced at the beginning of sporulation in *B. subtilis* (33, 72). Although the orthologs of these kinases are not conserved in clostridia, histidine kinases directly activated the Spo0A protein and induced sporulation in several clostridia (34–36). Thus, we focused on the gene expression of orphan histidine kinases in our microarray analysis. However, none of the orphan kinases reached the threshold defined by us in response to DCA (Table 2). Eswaramoorthy et al. (80) demonstrated that a less than 2-fold change in the concentration of KinA led to a 10-fold change in the number of spores. Thus, although we did not detect the upregulation of orphan kinases, they may still be involved in the sporulation process. DCA might enhance Spo0A activity by posttranslationally activating kinase function.

A second possibility is that DCA as a ligand stimulates a two-component system, leading to the activation of Spo0A. In the present study, we showed that DCA increased the expression of *nagH*, encoding hyaluronidase (μ -toxin) (Fig. 4; also see Table S7 in the supplemental material). Hiscox et al. (58, 59) revealed that *nagH* was positively regulated by the response regulator RevR in strain 13. They also showed that genes involved in the formation of the spore coat, septum, and cortex were downregulated in the *revR* null mutant (58). A total of 46% (30 out of the 65 orthologs identified) of RevR positively regulated genes in strain 13 (58) were upregulated during DCA-induced sporulation in this study (see Table S4), including genes specifically upregulated by DCA, *arcA*, *arcD*, *arcC*, AC7_2228 (nucleoside diphosphate kinase), and AC7_0585 (ABC transporter) (Fig. 4), while the expression of the ortholog of *revR* (AC7_0656) was not altered (see Table S3). The sensor kinase of RevR has not yet been reported; however, NCTC8239 may sense DCA by the two-component system involving RevR and regulate sporulation.

The third possibility is that DCA stimulates an unknown cascade that leads to the activation of Spo0A. Our microarray data at 3 hpi revealed a candidate for the activation of Spo0A: the phosphotransbutyrylase gene (AC7_A0093). This enzyme catalyzes the conversion of butyryl-CoA to BuP (74). High levels of BuP induced by the defective mutant of butyrate kinase, which converts BuP to butyrate, have been shown to accelerate sporulation in *C.*

acetobutylicum (33). In *Escherichia coli*, another phosphate, acetyl phosphate, can donate its phosphoryl group to the two-component response regulator (81). Thus, BuP may act as a phosphodonor for transcription factors. It is noteworthy here that AC7_A0093 has a Spo0A box in its promoter region (75), indicating functional similarities to the phosphotransbutyrylase with Spo0F, which is known to act as a phosphodonor for Spo0A in *Bacillus* spp. The corresponding gene, *spo0F*, possesses an Spo0A-binding site on its promoter region, and the transcription of *spo0F* is controlled by Spo0A~P in *B. subtilis* (82, 83), which supports this hypothesis. Thus, DCA may increase the production of BuP as a phosphodonor for the Spo0A protein in NCTC8239. Furthermore, the genes specifically upregulated by DCA as shown in Fig. 4 may be essential molecules in DCA-induced sporulation. Their involvement in the sporulation process needs to be investigated by, for example, gene knockout in future studies. We also propose other possibilities for the activation of Spo0A: (i) the factor(s) that senses DCA and induces the phosphorylation of Spo0A is not differentially expressed transcriptionally in response to DCA, or (ii) residual genes in the gaps between contigs are responsible for inducing the phosphorylation of Spo0A. Further investigations, including random mutagenesis and/or transcriptome sequencing (RNA-seq), will be needed in order to identify the bacterial factor(s) and elucidate the molecular mechanisms underlying DCA-induced sporulation.

Our microarray data revealed that several genes considered to be downstream of Spo0A in the sporulation signal cascade were not upregulated during DCA-induced sporulation (Fig. 5C and D; also see Table S8 in the supplemental material). These results were unexpected, because Galperin et al. (38) found that *spoIID*, *spoIIR*, *spoIVB*, *spoVK*, *spoVS*, *spoVG*, and *ylbJ* were essential for the sporulation of *B. subtilis* and were conserved in all spore-forming bacilli and clostridia. A transcriptome analysis during sporulation in SM101 revealed the upregulation of *spoIID*, *spoIVB*, *spoIVFB*, *spoVAC*, *spoVT*, *cotJC*, and *ylbJ* (32). Although the actual involvement of these genes in the sporulation of NCTC8239 remains to be delineated, a feasible explanation of our results would be differential sensitivity to our microarray analyses relative to others, so that the genes described above were expressed at levels below the limits of detection. Another possibility is that the genes are not involved in DCA-induced sporulation or that an alternative signaling molecule(s) plays a part in the DCA-stimulated sporulation cascade in NCTC8239.

As shown in Fig. S4 in the supplemental material, qRT-PCR confirmed that *spoIIR* was not upregulated during DCA-induced sporulation in NCTC8239. This observation implies that *spoIIR* is regulated by a factor separate from Spo0A and SigF. These results contrast with those for *B. subtilis* (38) and *C. difficile* (84) and suggest that *spoIIR* would be variably regulated in the *Firmicutes*.

It is becoming increasingly obvious that enteric pathogens use bile as an environmental cue to control the regulation of virulence genes (17–20). In the present study, DCA induced sporulation and the expression of *cpe*, encoding enterotoxin (Fig. 7B), which is consistent with our previous findings showing that DCA enhanced the production of CPE (25). In contrast, taurocholate was identified as a germinant for *C. difficile* (85, 86), which implies that different species of clostridia share the same stimuli in order to respond to opposite outcomes, sporulation and germination. In contrast to *C. difficile*, *C. perfringens* is a foodborne pathogen that has to establish an infection site in the intestinal tract. *C. perfrin-*

gens may have acquired a unique mechanism to respond to host factor(s) such as bile in order to survive and exert its pathological effects in the intestinal tract.

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