CodY Promotes Sporulation and Enterotoxin Production by *Clostridium perfringens* Type A Strain SM101

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ABSTRACT *Clostridium perfringens* type D strains cause enterotoxemia and enteritis in livestock via epsilon toxin production. In type D strain CN3718, CodY was previously shown to increase the level of epsilon toxin production and repress sporulation. *C. perfringens* type A strains producing *C. perfringens* enterotoxin (CPE) cause human food poisoning and antibiotic-associated diarrhea. Sporulation is critical for *C. perfringens* type A food poisoning since spores contribute to transmission and resistance in the harsh food environment and sporulation is essential for CPE production. Therefore, the current study asked whether CodY also regulates sporulation and CPE production in SM101, a derivative of *C. perfringens* type A food-poisoning strain NCTC8798. An isogenic *codY*-null mutant of SM101 showed decreased levels of spore formation, along with lower levels of CPE production. A complemented strain recovered wild-type levels of both sporulation and CPE production. When this result was coupled with the earlier results obtained with CN3718, it became apparent that CodY regulation of sporulation varies among different *C. perfringens* strains. Results from quantitative reverse transcriptase PCR analysis clearly demonstrated that, during sporulation, *codY* transcript levels remained high in SM101 but rapidly declined in CN3718. In addition, *abrB* gene expression patterns varied significantly between *codY*-null mutants of SM101 and CN3718. Compared to the levels in their wild-type parents, the level of *abrB* gene expression decreased in the CN3718 *codY*-null mutant strain but significantly increased in the SM101 *codY*-null mutant strain, demonstrating CodY-dependent regulation differences in *abrB* expression between these two strains. This difference appears to be important since overexpression of the *abrB* gene in SM101 reduced the levels of sporulation and enterotoxin production, supporting the involvement of AbrB repression in regulating *C. perfringens* sporulation.

KEYWORDS *Clostridium perfringens*, food poisoning, enterotoxin, sporulation, CodY, gene regulation

The Gram-positive, anaerobic sporeformer *Clostridium perfringens* is an important human and livestock pathogen, causing both histotoxic infections, such as gas gangrene, and intestinal infections, including enteritis and enterotoxemia (which involves toxins produced in the intestines being absorbed into the circulation so that they damage internal organs, like the brain, kidneys, or lungs) (1–4). The pathogenic versatility of *C. perfringens* largely involves its ability to produce ~20 different toxins. However, individual strains of this bacterium never express this entire toxin battery, which permits the classification of *C. perfringens* strains into five toxin types (types A to E) on the basis of the production of four typing toxins (2, 5, 6). All five types produce *C. perfringens* alpha toxin (CPA). In addition, type B strains make *C. perfringens* beta toxin (CPB) and *C. perfringens* epsilon toxin (ETX); type C strains produce CPB; and type D strains make ETX, which is an NIAID class B priority agent (4, 5).

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Each *C. perfringens* type causes specific diseases, mainly via the production of different toxins, including some that are not currently used for toxin typing, despite their proven biomedical importance. Foremost among those toxins is *C. perfringens* enterotoxin (CPE), which is produced by some *C. perfringens* type A strains and which causes food poisoning in humans (7, 8). This foodborne disease is the 2nd most common bacterial foodborne illness in the United States, where it affects about 1 million people per year and causes economic losses exceeding \$310 million per year (7–9). CPE-producing type A strains also cause 5 to 15% of all antibiotic-associated diarrhea cases (7, 10). Our lab fulfilled molecular Koch's postulate analyses to demonstrate that CPE production is necessary for CPE-producing type A strains to induce a gastrointestinal pathology in rabbit small intestinal loops (11). We also showed that purified CPE alone is sufficient to damage the small intestine and colon of experimental animals (11, 12).

Sporulation plays a critical role in many diseases caused by *C. perfringens*, especially *C. perfringens* type A food poisoning (7, 13). During this type of food poisoning, CPE is expressed only when *C. perfringens* sporulates in the intestines (7, 13). The enterotoxin is then released into the lumen when the sporulating mother cell lyses to release its mature spore (7). In addition, spores are important for the transmission of *C. perfringens* type A food poisoning, particularly since most strains causing food poisoning produce exceptionally resistant spores that facilitate survival under incomplete cooking conditions or in the presence of low temperatures (e.g., refrigeration or freezing) or food preservatives (7, 13).

Given the public health impact of *C. perfringens* type A food poisoning, it is very important to understand how *C. perfringens* regulates sporulation and CPE production. Previous studies demonstrated that Spo0A, the master regulator of sporulation, is essential for the sporulation of this bacterium (14). In addition, the conserved sporulation pathway downstream of the Spo0A master regulator, including four alternative sigma factors (SigF, SigE, SigK, and SigG), was shown to be critical for *C. perfringens* sporulation (15, 16). However, only three of those four sigma factors (the exception being SigG) are needed for CPE production (15). Currently, much less is known about the regulation of early events in *C. perfringens* sporulation.

We reported recently that, in *C. perfringens* type D strain CN3718, the CodY protein regulates ETX production (17). Contrary to its function in *Clostridium difficile* or *Staphylococcus aureus* (18–20), where CodY represses toxin production, the CodY protein of *C. perfringens* was shown to increase the level of ETX production by CN3718 (17). However, as is true for other Gram-positive bacteria (21, 22), *C. perfringens* CodY DNA binding was stimulated by GTP and branched-chain amino acids (17). By electrophoretic mobility shift assay, we also demonstrated that the CodY protein binds specifically to sequences upstream of the *etx* gene, suggesting direct CodY regulation of *etx* transcription (17). Despite its unexpected effect on ETX production, CodY repressed sporulation in this strain, as it does in *C. difficile* and *Bacillus* spp. (17, 23, 24). Specifically, an isogenic *codY*-null mutant of CN3718 was shown to make significantly more spores than wild-type strain CN3718 or a complemented strain, although the spores of that *codY*-null mutant germinated less well than the spores made by the wild-type or complemented strain (17).

Given the critical role of sporulation in type A food poisoning, the current study investigated whether CodY plays a role in regulating sporulation and CPE production by SM101, which is a transformable derivative of a *C. perfringens* type A food-poisoning strain (15, 16, 25). Furthermore, we compared the role of CodY in regulating sporulation by type A strain SM101 and type D strain CN3718 to better understand the potential diversity in the regulation of the sporulation process among *C. perfringens* strains.

RESULTS

Construction and characterization of an SM101 *codY*-null mutant and a complemented strain. Our previous work (17) showed that CodY represses sporulation in CN3718, a type D intestinal disease strain that produces epsilon toxin (ETX) during

vegetative growth. In contrast, *C. perfringens* type A food-poisoning strains produce *C. perfringens* enterotoxin (CPE), but only during sporulation (7, 13). Given the biomedical importance of this type of food poisoning (see the introduction), the current study investigated whether CodY also affects sporulation and, by extension, CPE production in type A food-poisoning strains of *C. perfringens*.

A *Clostridium*-modified TargetTron-mediated insertional mutagenesis system (26) was used to inactivate the *codY* gene in SM101, a transformable derivative of type A food-poisoning strain NCTC8798 (25). To confirm the construction of an SM101 *codY*-null mutant strain, PCR was performed using primers specific for the internal region of the *codY* open reading frame (ORF), which was targeted for intron insertion. Those internal PCR primers amplified a *codY*-specific PCR product of ~300 bp from wild-type strain SM101 DNA (Fig. 1A). Due to the targeted insertion of a 900-bp intron into the *codY* ORF, the same oligonucleotide primers amplified an ~1,200-bp PCR product when DNA from the SM101 *codY*-null mutant, named SM101::*codY*, was used (Fig. 1A). Using a DNA probe specific for the intron, Southern blot analyses then confirmed that DNA isolated from SM101::*codY* but not that isolated from wild-type strain SM101 carried an intron insertion. Furthermore, only one band was observed on this blot, indicating that only a single intron had inserted into SM101::*codY* (Fig. 1B).

Next, a *codY*-complemented SM101 strain, named SM101*codY*comp, was prepared by electroporation of a plasmid named pJIR750*codY*comp, which carries the entire wild-type *codY* gene, into *codY*-null mutant strain SM101::*codY*. Successful introduction of *codY* into the complemented strain was confirmed by PCR using our internal *codY*-specific primers, which produced both the ~300-bp band, indicating the presence of the wild-type *codY* gene, and an ~1,200-bp band, indicating the presence of the *codY* gene with an ~900-bp intron insertion (Fig. 1A).

To further characterize the putative SM101::*codY* and SM101*codY*comp strains, a CodY Western blot assay was performed using pelleted bacteria from 5-h SFP (17) vegetative cultures. As expected, both wild-type strain SM101 and SM101*codY*comp produced the 25-kDa CodY, while no CodY production by SM101::*codY* was detected (Fig. 1C). SM101*codY*comp produced less CodY protein than wild-type strain SM101, possibly due to the location of the *codY* gene, i.e., plasmid carriage of a chromosomal gene.

Finally, the vegetative growth phenotype of wild-type strain SM101, SM101::*codY*, and SM101*codY*comp in SFP medium was characterized. By measuring the change in the optical density at 600 nm (OD₆₀₀) over time, it was determined that the vegetative growth of these three strains was similar (Fig. 1D). However, the *codY*-null mutant showed a slightly lower level of growth than wild-type strain SM101 or SM101*codY*comp (Fig. 1D).

Effects of a *codY*-null mutation on sporulation and CPE production. CodY was first reported to repress sporulation in *Bacillus subtilis* (24). Recently, the role of CodY in regulating sporulation in *Clostridium* species has also come under study (17, 23). Our group demonstrated that CodY represses sporulation in *C. perfringens* type D strain CN3718 (17). Similarly, the McBride group showed that CodY represses sporulation in two *Clostridium difficile* strains (23). However, the extent of this repression differed significantly between *C. difficile* strains, indicating that the effects of CodY on gene regulation can vary according to the strain background (23).

Therefore, the current study sought to assess the involvement of CodY in regulating sporulation and CPE production by a *C. perfringens* food-poisoning strain. For this purpose, the SM101, SM101::*codY*, and SM101*codY*comp strains were grown overnight (~16 h) in Duncan-Strong (DS) sporulation medium. When the spores in those overnight cultures were enumerated, SM101::*codY* produced significantly fewer spores than wild-type strain SM101. This 1,000-fold sporulation defect was reversed by complementation of the mutant to restore CodY production (Fig. 2A). SM101::*codY* did not simply have delayed sporulation since the level of sporulation of this strain did not increase further when it was grown in DS medium for 3 days (data not shown).

FIG 1 Construction and characterization of an SM101 *codY*-null mutant and complemented strain. (A) PCR assay confirmation of the construction of an isogenic *codY*-null mutant or complemented strain. Using DNA from wild-type strain SM101, a PCR was performed using internal *codY* gene primers; this assay amplified the expected product of ~300 bp. However, using template DNA isolated from the *codY*-null mutant strain, which has an ~900-bp intron insertion in the *codY* gene, the same PCR assay amplified a product of ~1,200 bp. As expected, the complemented strain amplified both an ~300-bp product and an ~1,200-bp product when the same primers were used, indicating the presence of a wild-type and disrupted *codY* gene in this strain. The numbers at the left show the migration of DNA size makers, determined using a 100-bp molecular ruler (Fisher Scientific Company). (B) Southern blot hybridization of an intron-specific probe with DNA from wild-type strain SM101 or the *codY*-null mutant. DNA from each strain was digested with *EcoRI*, and the size of the probe-reactive DNA fragment is shown at the left. (C) Western blot analysis for CodY expression in pelleted cells from 5-h SPF medium cultures of wild-type strain SM101, *codY*-null mutant SM101::*codY*, and the complemented strain SM101*codY*comp. The size of the protein is shown at the left. (D) Postinoculation changes in the OD₆₀₀ for cultures of wild-type strain SM101, the *codY*-null mutant, and the complemented strain growing in SFP vegetative growth medium at 37°C. Every 2 h up to 8 h, a 1-ml aliquot of the SFP culture was removed and the OD₆₀₀ was determined.

Since the results in Fig. 2A were opposite our earlier results obtained with CN3718 (17), where CodY repressed sporulation, we next asked if SM101::*codY* simply grows poorly in DS medium. When the growth of the strains in DS medium was compared, only a small reduction in growth for SM101::*codY* compared with that for wild-type strain SM101 or strain SM101*codY*comp was detected (Fig. 2B). Because of that small difference in growth for SM101::*codY*, an experiment was performed to address the possibility that this mutant does not consume nutrients to the same extent as SM101, which could reduce its level of sporulation. For this purpose, early-stationary-phase cells of a 4-h culture of SM101::*codY* in DS medium were incubated in filter-sterilized supernatant collected from an early-stationary-phase (4-h) SM101 culture. However, even when this more depleted medium was used, SM101::*codY* cultures still developed only $\sim 1 \times 10^4$ spores/ml after overnight incubation.

FIG 2 Comparison of spore formation, growth, and CPE production in DS medium cultures of SM101, an isogenic *codY*-null mutant, and the complemented strain. (A) Quantification of heat-resistant spore formation by the wild-type, *codY*-null mutant, and complemented strains in DS medium. The bacteria were cultured in DS medium overnight (~16 h) at 37°C; those overnight cultures were then heat shocked for 20 min at 70°C. After 10-fold serial dilution with distilled water, the heat-shocked cultures were plated onto BHI agar plates and grown anaerobically overnight at 37°C for colony counting. Shown are the average results from three repetitions (log₁₀ scale). Error bars depict standard deviations. *, $P < 0.05$ compared to the wild-type strain by ordinary one-way ANOVA. (B) Comparison of postinoculation changes in the OD₆₀₀ for DS medium cultures of wild-type strain SM101, the *codY*-null mutant, and the complemented strain. Every 2 h up to 8 h, a 1-ml aliquot of the DS medium culture at 37°C was removed and its OD₆₀₀ was determined. (C) Detection of GUS activity levels in the SM101 wild-type and *codY*-null mutant strains transformed with a reporter plasmid where GUS production is driven from the promoter of the *spolIA* operon, which encodes SigF. The bacteria were grown in DS medium overnight (~16 h) at 37°C; GUS activity was then detected using the overnight culture supernatant. *, $P < 0.05$ compared to the wild-type strain by Student's *t* test. (D) Western blot results for CPE production by wild-type strain SM101, the SM101::codY mutant, and the complemented strain SM101codYcomp when cultured overnight in DS medium. The size of the protein is shown at the left.

Because the results presented in Fig. 2A indicated that CodY significantly enhances the sporulation of *C. perfringens* strain SM101, an experiment was performed to evaluate when this regulation occurs (i.e., during early or late sporulation). SigF, a key early sporulation-specific sigma factor, is encoded in the tricistronic *spolIA* operon (15). Therefore, wild-type strain SM101 and SM101::codY were transformed with a reporter plasmid where *gusA* expression is driven by the promoter controlling expression of the *spolIA* operon. After overnight (~16 h) growth in DS medium, the β -glucuronidase (GUS) activity in SM101 was significantly higher than the GUS activity in SM101::codY (Fig. 2C), indicating that CodY affects sporulation during the early stages of sporulation.

Western blotting to analyze CPE production in DS medium cultures was performed since CPE is produced only during *C. perfringens* sporulation (13, 15). Consistent with its reduced ability to make spores, as shown in Fig. 2A, the amount of CPE in DS medium cultures produced by the SM101::codY mutant was much less than that produced by wild-type strain SM101, and this effect was reversible by complementation (Fig. 2D). Taken together, the results presented in Fig. 2 indicate that CodY is important for sporulation and CPE production by *C. perfringens* type A food-poisoning strain SM101.

Differences in CodY regulatory effects between SM101 and CN3718. Given the differences between the current results obtained with SM101 and the previous results obtained with CN3718 (17) regarding the role of CodY in regulating sporulation, the growth and sporulation in DS medium of these two strains, their *codY*-null mutants, and their *codY*-null mutant *codY*-complemented strains were directly compared. The results (Fig. 3A) demonstrated that the growth rates of the CN3718::codY and *codY*-complemented strains in DS medium were not appreciably different from the growth rate of wild-type strain CN3718, which was consistent with the findings of our previous

FIG 3 Direct comparison of spore formation and growth by DS medium cultures of the SM101 and CN3718 wild-type strains and their *codY*-null mutant and complemented strains. (A) Postinoculation change in the OD₆₀₀ of cultures of wild-type strains SM101 and CN3718, their isogenic *codY*-null mutants (SM101::codY and CN3718::codY), and their complemented strains cultured in DS medium at 37°C. Every 2 h after inoculation up to 8 h, a 1-ml aliquot of a DS medium culture was removed and the OD₆₀₀ was read. (B) Quantification of heat-resistant spore formation by the wild-type, *codY*-null mutant, and complemented strains of SM101 or CN3718. The bacteria were cultured in DS medium overnight (~16 h) at 37°C; the overnight cultures were then heat shocked for 20 min at 70°C. After 10-fold serial dilution with distilled water, the heat-shocked cultures were plated onto BHI agar plates and grown anaerobically overnight at 37°C for colony counting. Shown are the average results from three repetitions (log₁₀ scale). Error bars depict standard deviations. *, *P* < 0.05 compared to each wild-type strain by ordinary one-way ANOVA. (C) Time course qRT-PCR analyses of *codY* transcription were performed with 20 ng of RNA isolated from 2-, 4-, and 6-h DS medium cultures of wild-type strain SM101 or CN3718. Average *C_T* values were normalized to the value for the housekeeping 16S RNA gene, and the fold differences were calculated using the comparative *C_T* (2^{-ΔΔC_T}) method. The value of each bar indicates the calculated fold change relative to the value for the 2-h culture. Shown are the mean values from three independent experiments. *, *P* < 0.05 compared to the 2-h culture by ordinary one-way ANOVA; **, *P* < 0.001 compared to the 2-h culture by ordinary one-way ANOVA.

studies (17). As also noted in Fig. 2, the SM101::codY mutant grew modestly more slowly than wild-type strain SM101 or the SM101 *codY*-complemented strain (Fig. 3A).

In contrast to the current results, where inactivation of the *codY* gene in type A strain SM101 decreased the level of sporulation compared to that of the wild-type or the *codY*-complemented strain, the results presented in Fig. 3 confirmed those presented in our previous report (17) that sporulation increases when *codY* is insertionally inactivated in type D strain CN3718. In both strains, the CodY sporulation phenotype effect was reversible by complementation (Fig. 3B).

The differences in sporulation between *codY*-null mutants of SM101 and CN3718 could involve a variation in *codY* expression kinetics between these two strains. Therefore, quantitative reverse transcriptase PCR (qRT-PCR) was performed to measure *codY* transcript levels when these strains were cultured in DS medium. Interestingly, in wild-type strain SM101, *codY* transcript levels remained constant between 2 and 6 h of culture in DS medium, whereas in CN3718, *codY* transcript levels decreased over the same culture time, an effect in which the difference in the results between these time points reached statistical significance (Fig. 3C). Collectively, the results presented in Fig. 3 suggest that the sustained presence of *codY* transcripts is important for SM101 but not CN3718 entry into sporulation and that differences in sporulation between these two strains may involve *codY* transcript levels.

FIG 4 Quantitative RT-PCR analysis for *abrB* and *virX* gene expression in SM101 or CN3718 wild-type, *codY*-null mutant, and complemented strains. Quantitative RT-PCR analyses of *abrB* (A) and *virX* (B) transcript levels were performed with 20 ng of the RNA isolated from 2-h DS medium cultures of the wild-type, *codY*-null mutant, and complemented strains. Average C_T values were normalized to the value for the housekeeping 16S RNA gene, and the fold differences were calculated using the comparative C_T ($2^{-\Delta\Delta C_T}$) method. The value of each bar indicates the calculated fold change relative to the value for the wild-type strains. Shown are the mean values from three independent experiments. *, $P < 0.05$ compared to the wild-type culture by ordinary one-way ANOVA; **, $P < 0.001$ compared to the wild-type culture by ordinary one-way ANOVA.

CodY regulates expression of the putative sporulation repressor gene *abrB* differently in SM101 than CN3718. To date, several genes that repress sporulation have been identified in Gram-positive spore-forming bacteria, including *abrB* in *Bacillus subtilis* and *virX* in *C. perfringens* (27, 28). To begin investigating how CodY differentially affects sporulation between these two strains, we compared the expression of the *abrB* and *virX* genes in 2-h DS medium cultures of SM101 and CN3718, as well as in cultures of their isogenic *codY*-null mutants and their *codY*-complemented strains. Results from qRT-PCR analyses revealed that *abrB* transcript levels were significantly higher in the SM101::codY mutant than in wild-type strain SM101 and its *codY*-complemented strain. In contrast, *abrB* transcript levels were significantly lower in CN3718::codY than in wild-type strain CN3718 and its *codY*-complemented strain (Fig. 4A).

A previous study reported that the *virX* gene, which encodes a small regulatory RNA, represses SM101 sporulation (28), possibly by decreasing the stability of RNA transcribed from genes encoding positive regulators (such as Spo0A and sigma factors) of *C. perfringens* sporulation. To examine whether CodY affects *virX* expression in DS medium cultures of SM101 and CN3718, qRT-PCR was performed. Those analyses detected no statistically significant difference in *virX* transcript levels among the wild-type, *codY*-null, and *codY*-complemented strains of SM101 or CN3718 (Fig. 4B).

Time course of *abrB* gene expression in DS medium cultures of both SM101 and CN3718. In *B. subtilis*, the AbrB protein acts to repress stationary-phase and

FIG 5 Quantitative RT-PCR time course study of *abrB* gene expression in DS medium cultures of wild-type strain SM101 or CN3718 and their *codY*-null mutant strains. Quantitative RT-PCR analyses of *abrB* transcript levels were performed using 20 ng of RNA isolated from 2-h, 4-h, and 6-h DS medium cultures of the wild-type strain (A) or the *codY*-null mutant (B). Average C_T values were normalized to the value for the housekeeping 16S RNA gene, and the fold differences were calculated using the comparative C_T ($2^{-\Delta\Delta C_T}$) method. The value of each bar indicates the calculated fold change relative to the value for the 2-h DS medium culture of that strain. Shown are mean values from three independent experiments. **, $P < 0.001$ compared to the wild-type culture by ordinary one-way ANOVA.

sporulation genes during exponential growth (27). In that bacterium, AbrB can repress the expression of positive sporulation regulators, such as SigH, as well as repress its own transcription (29). The *C. perfringens* genome encodes an *abrB* gene, but there are not yet any experimental results linking this gene to the regulation of *C. perfringens* sporulation (30, 31).

The results presented in Fig. 4A indicated that, for both SM101 and CN3718, *abrB* transcript levels significantly differed between the *codY*-null mutants and the wild-type or *codY*-complemented strain, with those differences correlating with the effects of a *codY*-null mutation on the levels of spore formation by each strain. Therefore, qRT-PCR was used to survey *abrB* expression during the first 6 h of culture in DS medium. For wild-type strains SM101 and CN3718 and their *codY*-null mutants, *abrB* expression increased between time zero and 2 h (data not shown), likely because the stationary-phase vegetative cells in the fluid thioglycolate (FTG) medium inoculum entered log-phase growth when they were cultured in fresh DS medium (Fig. 2B). However, for both wild-type strain SM101 and wild-type strain CN3718, *abrB* expression levels then significantly decreased after 2 h in DS medium (Fig. 5A). When *abrB* transcript levels, determined by qRT-PCR, in the SM101 or CN3718 *codY*-null mutant cultured in DS medium were similarly compared, *abrB* transcript levels remained stationary at between 2 and 6 h for SM101::*codY*, but those levels significantly decreased after 2 h in CN3718::*codY* (Fig. 5B).

The results from the experiments whose results are presented in Fig. 4 and 5 indicated that decreases in *abrB* transcript levels correlate with increased levels of sporulation by *C. perfringens*. To test if sequence variations upstream of the *abrB* ORF in CN3718 versus SM101 might mediate the different effects of CodY on *abrB* transcript levels in those two strains, two GUS reporter constructs were constructed, where *gusA* ORF expression was driven from 617 bp of the sequence directly upstream of either the SM101 *abrB* ORF or the CN3718 *abrB* ORF. Note that the sequences upstream of *abrB* in SM101 and CN3718 share 92.2% nucleotide sequence identity.

FIG 6 Comparison of GUS reporter activity driven by the SM101 *abrB* promoter or the CN3718 *abrB* promoter in DS medium cultures of wild-type strain SM101 or the SM101 *codY*-null mutant. GUS activity was detected in the SM101 wild-type strain and the *codY*-null mutant transformed with a plasmid carrying the *gusA* ORF fused with the SM101 *abrB* promoter or the CN3718 *abrB* promoter. The bacteria were grown in DS medium for 4 h at 37°C. GUS activity was then detected using the bacterial sonicated supernatants (see Materials and Methods). *, $P < 0.05$ compared to the wild-type GUS activity by Student's *t* test.

When SM101abrBp-GUS or CN3718abrBp-GUS was transformed into SM101 or SM101::*codY*, all transformants showed GUS activity (Fig. 6). However, significantly more GUS activity was detected in DS medium cultures of SM101::*codY* than in those of wild-type strain SM101 when the strains were transformed with either reporter plasmid, consistent with the higher natural *abrB* expression levels noted earlier (Fig. 4) for DS medium cultures of the *codY*-null mutant of SM101 than wild-type strain SM101. These results presented in Fig. 6 demonstrate that differences in *abrB* expression between DS medium cultures of the SM101 and CN3718 *codY*-null mutants do not appear to involve sequence variations upstream of the *abrB* ORF in these strains.

Overexpression of AbrB inhibits SM101 sporulation and reduces CPE production. The results presented above were consistent with CodY regulating sporulation and CPE production in SM101, at least in part, by reducing the level of AbrB production. However, as mentioned earlier, there has been no experimental evidence implicating AbrB involvement in the regulation of either *C. perfringens* sporulation or CPE production. No attempt was made in this study to construct an *abrB*-null mutant to demonstrate the involvement of AbrB in sporulation since similar studies using *abrB*-null mutants of *Bacillus subtilis* showed that higher levels of AbrB repress sporulation but some AbrB is needed for sporulation (32).

Instead, to demonstrate AbrB involvement in controlling *C. perfringens* sporulation, AbrB was overexpressed from a multicopy plasmid in wild-type strain SM101; as a negative control, the shuttle plasmid alone (with no *abrB* insert) was also transformed into SM101. The results of an initial experiment confirmed that these two strains displayed similar patterns of growth in DS medium (Fig. 7A). Next, an *abrB* qRT-PCR was performed to demonstrate that the construct overexpressing *abrB* caused an elevation in the levels of the *abrB* transcript in DS medium cultures of SM101 (Fig. 7B). Finally, the levels of sporulation and CPE production in DS medium were determined as described above, and the results revealed that the SM101 transformant carrying the pJIR750-*abrB* vector overexpressing AbrB produced significantly fewer spores than the SM101 transformant carrying the vector control (Fig. 7C). Similarly, overexpression of the *abrB* gene also resulted in significantly decreased levels of both *cpe* transcription (Fig. 7B) and CPE production (Fig. 7D).

DISCUSSION

C. perfringens forms spores that facilitate the effective persistence of this bacterium both inside the host and in the environment (7). For CPE-positive type A food-poisoning strains, spore formation is particularly important since it enhances survival in the food environment, contributes to foodborne transmission, and is essential for CPE produc-

FIG 7 Characterization of *abrB* gene overexpression effects in DS medium cultures of SM101. (A) Postinoculation change in the OD₆₀₀ for DS medium cultures of SM101 transformed with the empty vector [SM101(pJIR750)] or SM101 transformed with an *abrB*-carrying plasmid [creating *abrB*-overexpressing strain SM101(pJIR750-abrB)]. Both strains were grown in DS medium at 37°C; every 2 h up to 8 h, a 1-ml aliquot of DS medium culture was removed and the OD₆₀₀ was determined. (B) Quantitative RT-PCR analyses of *abrB* and *cpe* transcription were performed using 20 ng of RNA isolated from 2-h DS medium cultures of wild-type strain SM101(pJIR750) or *abrB*-overexpressing strain SM101(pJIR750-abrB). Average *C_T* values were normalized to the value for the housekeeping 16S RNA gene, and the fold differences were calculated using the comparative *C_T* ($2^{-\Delta\Delta C_T}$) method. The value for each bar indicates the calculated fold change relative to the value for the wild-type culture. Shown are the mean values from three independent experiments. *, *P* < 0.05 compared to the wild-type culture by Student's *t* test. (C) Comparison of heat-resistant spore formation by control strain SM101(pJIR750) and *abrB*-overexpressing strain SM101(pJIR750-abrB). The bacteria were grown in DS medium overnight (~16 h) at 37°C. The overnight cultures were then heat shocked for 20 min at 70°C. After 10-fold serial dilution with distilled water, the heat-shocked cultures were then plated onto BHI agar plates and grown anaerobically overnight at 37°C for colony counting. Shown are the average results from three repetitions (log₁₀ scale). Error bars depict standard deviations. *, *P* < 0.05 compared to each wild-type strain by Student's *t* test. (D) (Top) Western blot analysis of CPE production by three randomly chosen SM101(pJIR750) colonies and three randomly chosen SM101(pJIR750-abrB) colonies grown in DS medium. (Bottom) Quantification of CPE production using ImageJ software. Shown are the mean averages for the three different colonies. Error bars depict standard deviations. *, *P* < 0.05 compared to each wild-type strain by Student's *t* test.

tion (7, 13). Despite this significance, the early regulatory events controlling *C. perfringens* sporulation remain unclear.

Helping to address this knowledge gap, we recently identified the global nutritional state regulator CodY to be a repressor of sporulation in *C. perfringens* type D strain CN3718; i.e., an isogenic *codY*-null mutant showed significantly increased levels of sporulation compared to wild-type strain CN3718 (17). An enhanced sporulation phe-

notype for the *codY*-null mutants has also been reported for *B. subtilis*, *Bacillus thuringiensis*, and *C. difficile* (22–24, 33). Interestingly, while disruption of the *codY* gene increased the level of sporulation in two strains of *C. difficile*, significant differences in the sporulation frequencies of those two *codY*-null mutants were noted; i.e., a *codY*-null mutant of a poorly sporulating strain exhibited a 2-fold increase in sporulation compared to that for its wild-type parent, while a *codY*-null mutant of a strongly sporulating strain exhibited 1,400-fold more sporulation than its wild-type parent (23).

Those results (23), obtained using *C. difficile* *codY*-null mutants, established that strain variations in the CodY regulation of sporulation can occur even within a clostridial species. Therefore, given the critical role of CodY in *C. perfringens* type A food poisoning, an isogenic *codY*-null mutant of SM101, which is a transformable derivative of *C. perfringens* food-poisoning strain NCTC8798, was generated in the current study (25) to determine whether CodY represses sporulation in this strain as it does in CN3718 (17). In surprising contrast to the results observed for *codY*-null mutants of other Gram-positive sporeformers (including *C. perfringens* strain CN3718) (17, 23, 24), the SM101 *codY*-null mutant showed a 3-log reduction in spore formation and a much lower level of CPE production than wild-type strain SM101. Those results obtained with the SM101 *codY*-null mutant revealed that CodY positively regulates sporulation and CPE production in SM101. Importantly, when the SM101 *codY*-null mutant was complemented to restore CodY production, the levels of both spore formation and CPE production increased, indicating that the mutant phenotype was not due to a secondary mutation in the SM101 *codY*-null mutant strain.

A *codY* qRT-PCR time course experiment then provided insights into the basis for the opposite role of CodY in regulating sporulation by SM101 versus CN3718. Those analyses detected significant differences in *codY* gene transcript levels between the two wild-type strains when they were cultured in DS sporulation medium. Specifically, *codY* transcript levels remained high at between 2 and 6 h in wild-type strain SM101 DS medium cultures, but the levels of this transcript decreased significantly during the same time period in CN3718 DS medium cultures. This result is consistent with our previous findings (17; this study) that CodY is a sporulation repressor in CN3718; i.e., the decrease in the level of *codY* transcription observed during culture of CN3718 in DS sporulation medium correlates with the increased rate of entry of that strain into sporulation. In contrast, the finding that the presence of steady-state *codY* transcript levels during the first 6 h of SM101 growth in DS medium, coupled with the observation that a *codY* SM101-null mutant makes significantly fewer spores than wild-type strain SM101, supports the suggestion that CodY is an important positive regulator of sporulation in this food-poisoning strain. Since sporulation is essential for CPE production, it was not surprising to find that CodY also positively regulates the production of this toxin, which is critically important for *C. perfringens* type A food poisoning (7, 11, 13). Future studies are needed to identify why *codY* transcript levels remain high in SM101 but fall dramatically in CN3718 when both strains are cultured in DS medium.

We then investigated the timing of CodY regulation of sporulation in SM101 using a reporter vector where GUS activity is driven by the promoter controlling expression of the *spoIIA* operon, which is expressed early during sporulation and encodes SigF (15). This experiment showed that sporulating cultures of the SM101 *codY*-null mutant had significantly lower levels of GUS activity than sporulating cultures of wild-type strain SM101 carrying the same vector. This finding indicates that, in wild-type strain SM101, CodY regulation occurs during the relatively early stages of sporulation, i.e., prior to the production of SigF, which is the first sporulation-associated alternative sigma factor made by *C. perfringens* (15).

To investigate potential mechanisms by which CodY might positively control sporulation in SM101, we evaluated the effects of CodY on the transcription of two genes, *virX* and *abrB*, which have been implicated as repressors of the initiation of sporulation by, respectively, *C. perfringens* or other Gram-positive sporeformers (27, 28). In *B. subtilis*, AbrB is a regulatory protein that principally antagonizes the sporulation of *B. subtilis* by repressing the gene encoding the sporulation master regulator Spo0A (34). However, at

FIG 8 Model for differential early spore regulation in SM101 versus CN3718. When SM101 enters sporulation, CodY expression is maintained and this protein mediates the reduced production of AbrB, which functions as a sporulation repressor. In contrast, when CN3718 enters sporulation, less CodY is made, also leading to smaller amounts of the AbrB sporulation repressor. Arrows, effects of a *codY*-null mutation. This model does not preclude the possibility that CodY also affects sporulation by other regulatory effects.

least some functional AbrB is needed for sporulation since this protein inhibits the phosphatase-encoding gene *spo0E*, which can dephosphorylate phosphorylated Spo0A and block sporulation (29). Carriage of the *abrB* gene is conserved among all *Bacillus*, *Clostridium*, *Geobacillus*, and *Listeria* species, and the AbrB protein of *C. perfringens* SM101 shares 51.5% amino acid sequence identity with *B. subtilis* AbrB. Nonetheless, the role of AbrB in regulating sporulation in *Clostridium* spp. has received little study to date, with the exception of one study using an antisense vector against *abrB* that produced, somewhat surprisingly, a delay in spore formation by *Clostridium acetobutylicum* (35).

The current study detected in both wild-type *C. perfringens* SM101 and wild-type *C. perfringens* CN3718 a decrease in *abrB* gene transcript levels at between 2 and 6 h of culture in DS sporulation medium. In contrast, significant differences in *abrB* transcript levels between the *codY*-null mutants of these two *C. perfringens* strains were detected over this time period when they were cultured in DS medium. While *abrB* transcript levels in the CN3718 *codY*-null mutant strain decreased after 2 h in DS medium, *abrB* transcript levels remained stationary at between 2 and 6 h of growth of the SM101 *codY*-null mutant in DS medium.

Those effects on *abrB* transcript levels suggested one insight into why CodY promotes the sporulation of SM101; i.e., CodY represses *abrB* transcript levels in SM101 but not CN3718. Therefore, an experiment tested whether the observed differences in the regulatory effects of CodY on *abrB* expression between SM101 and CN3718 might involve differences in their sequences upstream of their *abrB* ORFs. Using reporter vectors where GUS production is driven from the *abrB* promoter of either SM101 or CN3718, GUS activity in DS medium was similar when SM101 was transformed with either reporter plasmid. GUS activity was also similar for DS medium cultures of the SM101 *codY*-null mutant transformed with either reporter plasmid. However, GUS activity was significantly lower in wild-type strain SM101 than the SM101 *codY*-null mutant transformed with either reporter plasmid, supporting the suggestion that *abrB* expression differences between DS medium cultures of SM101 and CN3718 are independent of sequence variations upstream of the *abrB* ORF of these two strains. Whether CodY directly or indirectly affects *abrB* gene expression in SM101 will require further study, although it is notable that a predicted CodY binding box is located immediately upstream of the *abrB* ORF.

Considering the known involvement of AbrB in regulating sporulation in other bacteria (29, 35), the differences in CodY involvement in regulating *abrB* transcript levels observed in DS medium cultures of SM101 and CN3718 suggest a model (Fig. 8) where AbrB is also a sporulation repressor in *C. perfringens*. At between 2 and 6 h of culture in DS medium, CodY levels remain high and, at least in part, promote sporulation in SM101 by lowering the levels of AbrB. In contrast, CodY is not necessary to lower AbrB levels in CN3718 DS medium cultures. The regulator that reduces *abrB* transcript levels in sporulating CN3718 DS medium cultures remains to be identified. Note that this model does not preclude other, perhaps even more important, regulatory effects of CodY on sporulation.

The model shown in Fig. 8 depicts AbrB as a sporulation repressor in both SM101

and CN3718, but prior to the current study, there had been no experimental evidence implicating AbrB in the regulation of sporulation by *C. perfringens*. Another group recently performed SM101 whole-genome expression profiling during sporulation using DNA microarrays, but the *abrB* gene (CPR_0268) was missing from that group's array (31). Therefore, to explore if AbrB regulates sporulation in *C. perfringens*, our current study overexpressed AbrB in wild-type strain SM101, as confirmed by our qRT-PCR results. The results obtained support a role for AbrB in repressing sporulation since (i) overexpression of AbrB decreased the ability of SM101 to form spores or produce CPE, as expected of a repressor, and (ii) this AbrB overexpression did not affect growth in DS medium, supporting the suggestion that the effects of AbrB overexpression are specific for sporulation rather than global gene expression.

VirX is an RNA regulator that has been shown to reduce the level of sporulation by SM101 (28). Specifically, VirX acts by repressing genes encoding positive regulators (Spo0A and sigma factors) of *C. perfringens* sporulation. The current study determined by qRT-PCR that VirX is not part of the CodY regulon. Combining this result with the current results implicating AbrB in sporulation repression and previous results indicating that VirX is a sporulation repressor indicates that both CodY-dependent and CodY-independent repressors of early sporulation can exist in a single *C. perfringens* strain.

After spores are formed and released, it is important that they eventually germinate back to vegetative cells. Our previous study (17) showed that a CN3718 *codY*-null mutant was impaired for spore germination. Due to the very limited sporulation rate of the SM101 *codY*-null mutant, no attempt was made during the current work to evaluate the germination capability of this mutant.

In summary, the current study reports that CodY significantly promotes sporulation and CPE production by SM101, a transformable derivative of a typical *C. perfringens* food-poisoning strain. This work also showed that *abrB* is part of the CodY regulon in this strain and that AbrB levels can impact the levels of sporulation by SM101. Finally, this study identified diversity in the CodY regulation of sporulation among *C. perfringens* strains. In this regard, it is notable that typical type A food-poisoning strains, like SM101, are genotypically distinct from most other *C. perfringens* strains, as assessed by multilocus sequence type analyses of housekeeping genes (36). In addition, these typical food-poisoning strains carry a chromosomal *cpe* gene (rather than no *cpe* gene or a plasmid-borne *cpe* gene) but lack *pfo* and *nanI* genes, encoding perfringolysin O and NanI sialidase, respectively (30, 37). They also produce an unusual small acid-soluble protein 4 (SASP4) variant that results in extremely tough spores highly resistant to heat, cold, and food preservatives compared to the spores of most other *C. perfringens* strains (38). Their spore resistance benefits the survival of the food-poisoning strains in the food environment (7, 13).

While this study offers new insights into the regulation of sporulation and CPE production, particularly for food-poisoning strains, more work is clearly needed to fully understand the regulatory effects of CodY and AbrB, including the basis for the differences identified between SM101 and CN3718 with respect to control of the early steps in sporulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The *C. perfringens* strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5 α cells (New England BioLabs) were used as the cloning host.

The broth media used for culturing *C. perfringens* included fluid thioglycolate (FTG) medium (Becton Dickinson), SFP medium (17), TGY medium (17), and Duncan-Strong (DS) sporulation medium (39). Brain heart infusion (BHI) and SFP agar plates (Becton Dickinson) were used for *C. perfringens* culture on solid media. After inoculation with *C. perfringens*, the plates were incubated using a GasPak EZ anaerobe container system (BD). For culturing of *E. coli*, Luria-Bertani (LB) broth and agar (1.5% agar [Becton Dickinson]) were used. All antibiotics used were purchased from Fisher Scientific. All *C. perfringens* and *E. coli* strains were cultured at 37°C in this study.

Construction of an isogenic *codY*-null mutant and complemented strain of SM101. The *codY* gene in SM101 was inactivated by insertion of a targeted group II intron into the *codY* open reading frame (ORF) using a *Clostridium*-modified TargeTron system (26). Since the *codY* ORF sequences in SM101

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
SM101	Transformable derivative of NCTC8798, a CPE-positive <i>C. perfringens</i> type A human food-poisoning strain	25
SM101::codY	SM101 codY-null mutant	This study
SM101codYcomp	SM101 codY-complemented strain	This study
CN3718	ETX-positive <i>C. perfringens</i> type D animal disease strain	17
CN3718::codY	CN3718 codY-null mutant	17
CN3718codYcomp	CN3718 codY-complemented strain	17
SM101(pJIR750-abrB)	SM101 AbrB-overexpressing strain	This study
SM101(pJIR750)	SM101 with a plasmid as a control	This study
Plasmids		
pJIR750codYi	Construction of a codY-null mutant	17
pJIR750codYcomp	Complementation of codY-null mutant	17
pJIR750	<i>C. perfringens</i> - <i>E. coli</i> shuttle plasmid	40
pJIR750-abrB	Construction of an abrB-overexpressing strain	This study
pJIR750-PsigF-GusA	Vector where GUS expression is driven by the spoIIA promoter	This study
pJIR750-PabrB-GusA	abrB promoter-GUS expression vector	This study

and CN3718 share 100% nucleotide sequence identity (17), the current study employed the same codY-specific TargetTron plasmid (pJIR750codYi) that had been used to inactivate the codY gene in type D strain CN3718 (17). The preparation and selection of the SM101 codY-null mutant (named SM101::codY) were performed as described previously (17). The PCR primers codYKOF and codYKOR, which were described previously (17), were used for mutant screening by PCR.

The SM101 codY-null mutant was complemented by using the same codY-encoding plasmid, named pJIR750codYcomp (17), that had been used to complement the CN3718::codY mutant. Note that (i) pJIR750codYcomp carries the entire wild-type strain CN3718 codY gene, including the natural promoter and ribosome binding site, and (ii) the sequence of the codY gene (including the ORF and upstream and downstream flanking sequences) is 99% identical between SM101 and CN3718. To prepare a CodY-complemented strain, SM101::codY was transformed by electroporation with pJIR750codYcomp. The resultant complemented strain, named SM101codYcomp, was then selected on BHI agar plates containing 15 mg/liter of chloramphenicol. The complemented strain was confirmed by PCR and Western blot analysis, as described below.

Construction of an AbrB-overexpressing derivative of wild-type strain SM101. An AbrB-overexpressing strain was constructed by cloning the abrB ORF, flanked by ~600 bp of upstream sequence and ~250 bp of downstream sequence, into the pJIR750 *C. perfringens*-*E. coli* shuttle plasmid and then transforming this new plasmid into wild-type strain SM101. Briefly, DNA was isolated from wild-type strain SM101 using a MasterPure Gram-positive bacterial DNA purification kit (Epicentre, Madison, WI). PCR was then performed using LongAmp Taq DNA polymerase (New England BioLabs) and primers abrBcompF and abrBcompR (Table 2). The resultant 1,130-bp, gel-purified PCR product and the pJIR750 vector (40) (Table 1) were each then cut with EcoRI/PstI at 37°C for 1 h, according to the manufacturer's instructions (New England BioLabs). After ligation of the digested vector and PCR product using an instant sticky-end ligase master mix (New England BioLabs), the vector was directly transformed into wild-type strain SM101 by electroporation, and the AbrB-overexpressing strain, named SM101(pJIR750-abrB), was then selected on BHI agar plates containing 15 mg/liter of chloramphenicol.

TABLE 2 Primers used in this study

Primer name	Sequence ^a (endonuclease)	Purpose
abrBcompF	5'-ATTCgaattcATCACATTACAAGTTAATACCTTC-3' (EcoRI)	abrB overexpression
abrBcompR	5'-GCAGctgcagGAATGAAGAAAAAGATGCTATCAA-3' (PstI)	
qabrBF	5'-AATCTATGTAGATGGAGAGCAAAATC-3'	abrB qRT-PCR
qabrBR	5'-GTAGTTAACAACATCTCTAGCATCT-3'	
qvixXF	5'-ATGGAAGATAAAATTTAGTTTGT-3'	virX qRT-PCR
qvixXR	5'-TTTGAGCTTTTCTTGCTTTT-3'	
sigFF	5'-TTACgaattcGTGTAATGGATTGTCTGTTATAG-3' (EcoRI)	Promoter driving spoIIA expression
sigFR	5'-GGctgcaggatccATTTCCTATTTCTCAAAATCTAACCACAT-3' (PstI and BamHI)	
abrBpro-F	5'-ATTGgagctcATCACATTACAAGTTAATACCTTC-3' (SacI)	abrB promoter
abrBpro-R	5'-GCAGgtgcagTTTACAACCTTCGACAAAACGCAT-3' (Sall)	
gusAF	5'-CCGgattcATGTTACGTCCTGTAGAAACC-3' (BamHI)	gusA gene
	5'-CATCATgtcgacATGTTACGTCCTGTAGAAAC-3' (Sall)	
gusAR	5'-ATGCctgcagTCAATGTTTGCCTCCCT-3' (PstI)	

^aRestriction enzyme cut sites are shown in lowercase letters.

The phenotype of this strain was confirmed by *abrB* quantitative reverse transcriptase PCR (qRT-PCR) analysis, as described below.

PCR and Southern blot analyses of the SM101 *codY*-null mutant and complemented strain. DNA was isolated from wild-type strain SM101, the *codY*-null mutant SM101::*codY*, and the complemented strain SM101*codY*comp by using the MasterPure Gram-positive bacterial DNA purification kit. Using DNA samples from those strains, PCR was performed with primers *codY*KOF and *codY*KOR. Each reaction mixture was subjected to the following PCR amplification conditions: cycle 1, consisting of 95°C for 2 min; cycles 2 through 35, consisting of 95°C for 30 s, 55°C for 40 s, and 68°C for 80 s; and a final extension for 5 min at 68°C. An aliquot (20 μ l) of each PCR sample was electrophoresed on a 1.5% agarose gel and then visualized by staining with ethidium bromide.

For intron Southern blot analysis, aliquots of DNA (3 μ g) from SM101 and SM101::*codY* were digested overnight with *Eco*RI at 37°C, according to the manufacturer's instructions (New England BioLabs). Each digested DNA was then run on a 1% agarose gel. After alkali transfer to a nylon membrane (Roche Applied Science), the blot was hybridized with a digoxigenin-labeled, intron-specific probe, as described previously (17). CSPD substrate (Roche Applied Science) was used for detection of digoxigenin-labeled hybridized intron probes according to the manufacturer's instructions.

Western blot analyses of CodY and CPE production. For CodY Western blot analyses, 0.2-ml aliquots from an overnight FTG culture of SM101, SM101::*codY*, or SM101*codY*comp were inoculated into 10 ml of SFP medium. After incubation at 37°C for 5 h, each culture was adjusted to an equivalent optical density at 600 nm (OD_{600}) using a Bio-Rad SmartSpec spectrophotometer, and equal volumes of those adjusted cultures were centrifuged. Pelleted cells were then analyzed for CodY production by Western blotting using a rabbit polyclonal antiserum against *B. subtilis* CodY (17), kindly provided by Abraham Sonenshein.

For CPE Western blotting, a 0.2-ml aliquot of an overnight FTG culture of the wild-type, the *codY*-null mutant, the complemented strain, *AbiB*-overexpressing strain SM101(pJIR750-*abrB*), or control strain SM101(pJIR750) was added to 10 ml of freshly prepared DS or SFP medium. After culture overnight at 37°C, each culture was adjusted to an equivalent OD_{600} and equal volumes of those adjusted cultures were centrifuged. Equal volumes of the supernatants of those cultures were mixed with 5 $\times$ SDS loading buffer. Western blotting of those samples was then performed using a CPE anti-rabbit polyclonal antibody, as previously described (39).

Measurement of growth and sporulation. For analysis of growth in vegetative cultures, a 0.2-ml aliquot of an overnight FTG culture of the SM101, SM101::*codY*, or SM101*codY*comp strain was added to 10 ml of SFP medium. The cultures were incubated at 37°C, and every 2 h thereafter (up to 8 h), a 1-ml aliquot culture was collected and the OD_{600} was determined. For evaluation of growth in DS medium, a 0.2-ml aliquot of an overnight FTG culture of the SM101, SM101::*codY*, SM101*codY*comp, SM101(pJIR750), or SM101(pJIR750-*abrB*) strain was added to 10 ml of DS medium. Those cultures were incubated at 37°C, and every 2 h thereafter (up to 8 h), a 1-ml aliquot culture was collected and the OD_{600} was measured.

To enumerate spore formation, a 0.2-ml aliquot of an overnight FTG culture of the SM101, SM101::*codY*, SM101*codY*comp, SM101(pJIR750), or SM101(pJIR750-*abrB*) strain was added to 10 ml of DS medium (15, 17, 41). After overnight incubation at 37°C, each DS medium culture was heated at 70°C for 20 min to kill the remaining vegetative cells and enhance spore germination (41). Those heat-shocked suspensions were then serially diluted from 10^2 to 10^7 with sterile water and plated onto BHI agar plates. After incubation overnight at 37°C in an anaerobic jar, the colonies on each BHI agar plate were counted.

RNA extraction and qRT-PCR. A 0.2-ml aliquot from an overnight FTG culture of SM101, SM101::*codY*, SM101*codY*comp, CN3718, CN3718::*codY*, CN3718*codY*comp, SM101(pJIR750), or SM101(pJIR750-*abrB*) was added to 10 ml of DS medium. Total RNA was extracted from pelleted cells of 0-, 2-, 4-, or 6-h cultures (as specified above) using the saturated phenol (Fisher Scientific) method, as described previously (17). Purified RNA was quantified by determination of the absorbance at 260 nm and stored in a -80°C freezer. An aliquot (1 μ g) of RNA was first synthesized to cDNA using a Thermo Scientific Maxima first-strand cDNA synthesis kit for qRT-PCR, according to the manufacturer's instructions. Briefly a 20- μ l aliquot of the reaction mixture was amplified at 25°C for 10 min, then at 50°C for 30 min, and finally, at 85°C for 5 min. Before qRT-PCR, this cDNA was diluted 10 times to 5 ng/ μ l. All qRT-PCR primers were designed using the Integrated DNA Technologies (IDT) website. The qRT-PCR primers used to amplify the 16S RNA gene, *cpe* gene, and *codY* gene sequences were published previously (17, 37). The primers used for *abrB* qRT-PCR were *qabrBF* and *qabrBR* (Table 2), and those used for qRT-PCR amplification of *virX* transcripts were *qvixF* and *qvixR* (Table 2).

Power SYBR green PCR master mix (ThermoFisher Scientific) and a StepOnePlus qRT-PCR instrument (Applied Biosystems) were used to perform qRT-PCR. After qRT-PCR, the relative quantitation of mRNA expression was normalized to the level of constitutive expression of the housekeeping 16S RNA gene and calculated by the comparative threshold cycle (C_T ; $2^{-\Delta\Delta C_T}$) method (17).

Construction of GUS reporter vectors. To construct a β -glucuronidase (GUS) reporter vector where *gusA* is expressed from the promoter of the *spolIA* operon encoding SigF (15), ~ 800 bp of upstream sequence from the *spolIA* operon was amplified from *C. perfringens* strain SM101 using PCR primers *sigFF* and *sigFR*. The resulting PCR product was digested with *Eco*RI and *Pst*I and ligated into the *C. perfringens*-*E. coli* shuttle vector pJIR750 digested with the same enzymes to form vector pJIR750-*PsigF* (Table 1). This vector was then transformed into *E. coli* TOP10 cells (Invitrogen), and pJIR750-*PsigF* was harvested using a QIAprep Spin miniprep kit (Qiagen). The *gusA* gene was PCR amplified from *E. coli* TOP10 cells using PCR primers *gusAF* and *gusAR* (Table 2). The resulting PCR product was digested with *Bam*HI, run on an agarose gel, and gel extracted. *Pst*I was then used to cut the 3' end of the gene, and the resulting fragment was ligated into pJIR750-*PsigF* digested with *Bam*HI and *Pst*I before transforma-

tion of this plasmid into *E. coli* TOP10 cells. The resultant transformants were screened by PCR for the fragment extending from the 5' end of the *spolA* operon promoter to the 3' end of the *gusA* gene. Positive colonies were used to harvest pJIR750-*PsigF*-*GusA*, which was then electroporated into *C. perfringens* strain SM101 or the SM101 *codY*-null mutant.

To construct the *abrB* promoter-GUS reporter vector, 617 bp of upstream sequence from the *abrB*-containing operon was amplified from *C. perfringens* strain SM101 or CN3718 using PCR primers *abrB*pro-F and *abrB*pro-R (Table 2). The *gusA* gene was then amplified using PCR primers *gusA*F and *gusA*R (Table 2). These PCR products were digested with the appropriate restriction enzymes and then cloned stepwise into pJIR750 using the *SacI*, *Sall*, and *PstI* sites to create the reporter construct pJIR750-*PabrB*-*GusA*. This construct was then electroporated into *C. perfringens* strain SM101 or the SM101 *codY*-null mutant.

Measurement of GUS activity. To compare the relative expression from the promoter of the *spolA* operon encoding SigF using GUS reporter activity, SM101 and its isogenic *codY*-null mutant transformants carrying pJIR750-*PsigF*-*GusA* were grown overnight at 37°C in DS medium. The OD₆₀₀s of these overnight cultures were measured, and then the cultures were centrifuged and the supernatants were retained to measure GUS activity. To compare the relative expression from the *abrB* promoter, transformants of both SM101 and its isogenic *codY*-null mutant carrying the *abrB* promoter-GUS reporter vector were cultured for 4 h at 37°C in DS medium. After their OD₆₀₀s were measured, the cultures were centrifuged and the pellets of these cultures were resuspended in 1 ml of phosphate-buffered saline (PBS) buffer. The suspensions were then lysed on ice using a Qsonica sonicator (total run time, 2 min; maximum output, 30%) and centrifuged for 10 min.

Using those samples, GUS activity was measured by adding 50 µl of 6 mM 4-nitrophenyl-β-D-glucuronide (in PBS buffer) to 250 µl of the supernatant of an overnight culture (sigFp-GUS) or the lysed supernatant of cells in PBS buffer (*abrB*p-GUS). Following a 30-min or 60-min incubation at 37°C, the absorbance at 405 nm was read. The GUS activities were calculated and are provided as Miller specific activity units (42).

Statistical analysis. Student's unpaired *t* test (when comparing the results for two groups) and ordinary one-way analysis of variance (ANOVA; when comparing the results for more than two groups against the results for the wild type with Dunnett's *post hoc* test) were applied to test for statistically significant differences among the results.

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REFERENCES

- McClane BA, Uzal FA, Miyakawa MF, Lyerly D, Wilkins TD. 2006. The enterotoxigenic *Clostridium*, p 688–752. In Dworkin M, Falkow S, Rosenberg E, Schleifer H, Stackebrandt E (ed), *The prokaryotes*, 3rd ed. Springer, New York, NY.
- Li J, Adams V, Bannam TL, Miyamoto K, Garcia JP, Uzal FA, Rood JI, McClane BA. 2013. Toxin plasmids of *Clostridium perfringens*. *Microbiol Mol Biol Rev* 77:208–233. <https://doi.org/10.1128/MMBR.00062-12>.
- Songer JG. 1996. Clostridial enteric diseases of domestic animals. *Clin Microbiol Rev* 9:216–234.
- Uzal FA, Freedman JC, Shrestha A, Theoret JR, Garcia J, Awad MM, Adams V, Moore RJ, Rood JI, McClane BA. 2014. Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. *Future Microbiol* 9:361–377. <https://doi.org/10.2217/fmb.13.168>.
- Petit L, Gilbert M, Popoff M. 1999. *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiol* 7:104–110. [https://doi.org/10.1016/S0966-842X\(98\)01430-9](https://doi.org/10.1016/S0966-842X(98)01430-9).
- McDonel JL. 1986. Toxins of *Clostridium perfringens* types A, B, C, D, and E, p 477–517. In Dorner F, Drews H (ed), *Pharmacology of bacterial toxins*. Pergamon Press, Oxford, United Kingdom.
- McClane BA, Robertson SL, Li J. 2013. *Clostridium perfringens*, p 465–489. In Doyle MP, Buchanan RL (ed), *Food microbiology: fundamentals and frontiers*, 4th ed. ASM Press, Washington, DC.
- CDC. 2011. CDC estimates of foodborne illness in the United States: *Clostridium perfringens*. CDC, Atlanta, GA. <http://www.cdc.gov/foodborneburden/clostridium-perfringens.html>.
- Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M, Roy S, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg Infect Dis* 17:7–15. <https://doi.org/10.3201/eid1701.P11101>.
- Carman RJ. 1997. *Clostridium perfringens* in spontaneous and antibiotic-associated diarrhoea of man and other animals. *Rev Med Microbiol* 8(Suppl 1):S43–S45. <https://doi.org/10.1097/00013542-199712001-00023>.
- Sarker MR, Carman RJ, McClane BA. 1999. Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. *Mol Microbiol* 33:946–958. <https://doi.org/10.1046/j.1365-2958.1999.01534.x>.
- Garcia JP, Li J, Shrestha A, Freedman JC, Beingesser J, McClane BA, Uzal FA. 2014. *Clostridium perfringens* type A enterotoxin damages the rabbit colon. *Infect Immun* 82:2211–2218. <https://doi.org/10.1128/IAI.01659-14>.
- Li J, Paredes-Sabja D, Sarker M, McClane B. 2016. *Clostridium perfringens* sporulation and sporulation-associated toxin production. *Microbiol Spectr* 4:TBS-0022-2015. <https://doi.org/10.1128/microbiolspec.TBS-0022-2015>.
- Huang IH, Waters M, Grau RR, Sarker MR. 2004. Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. *FEMS Microbiol Lett* 233:233–240. <https://doi.org/10.1111/j.1574-6968.2004.tb09487.x>.
- Li J, McClane BA. 2010. Evaluating the involvement of alternative sigma factors SigF and SigG in *Clostridium perfringens* sporulation and enterotoxin synthesis. *Infect Immun* 78:4286–4293. <https://doi.org/10.1128/IAI.00528-10>.
- Harry KH, Zhou R, Kroos L, Melville SB. 2009. Sporulation and enterotoxin (CPE) synthesis are controlled by the sporulation-specific factors SigE and SigK in *Clostridium perfringens*. *J Bacteriol* 191:2728–2742. <https://doi.org/10.1128/JB.01839-08>.
- Li J, Ma M, Sarker MR, McClane BA. 2013. CodY is a global regulator of

- virulence-associated properties for *Clostridium perfringens* type D strain CN3718. *mBio* 4:e00770-13. <https://doi.org/10.1128/mBio.00770-13>.
18. Stenz L, Francois P, Whiteson K, Wolz C, Linder P, Schrenzel J. 2011. The CodY pleiotropic repressor controls virulence in gram-positive pathogens. *FEMS Immunol Med Microbiol* 62:123–139. <https://doi.org/10.1111/j.1574-695X.2011.00812.x>.
  19. Dineen SS, McBride SM, Sonenshein AL. 2010. Integration of metabolism and virulence by *Clostridium difficile* CodY. *J Bacteriol* 192:5350–5362. <https://doi.org/10.1128/JB.00341-10>.
  20. Majerczyk CD, Sadykov MR, Luong TT, Lee C, Somerville GA, Sonenshein AL. 2008. *Staphylococcus aureus* CodY negatively regulates virulence gene expression. *J Bacteriol* 190:2257–2265. <https://doi.org/10.1128/JB.01545-07>.
  21. Sonenshein AL. 2005. CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. *Curr Opin Microbiol* 8:203–207. <https://doi.org/10.1016/j.mib.2005.01.001>.
  22. Brinsmade SR. 15 October 2016. CodY, a master integrator of metabolism and virulence in Gram-positive bacteria. *Curr Genet*. <https://doi.org/10.1007/s00294-016-0656-5>.
  23. Nawrocki K, Edwards A, Daou N, Bouillaut L, McBride S. 2016. CodY-dependent regulation of sporulation in *Clostridium difficile*. *J Bacteriol* 198:2113–2130. <https://doi.org/10.1128/JB.00220-16>.
  24. Ratnayake-Lecamwasam M, Serror P, Wong KW, Sonenshein AL. 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev* 15:1093–1103. <https://doi.org/10.1101/gad.874201>.
  25. Zhao Y, Melville SB. 1998. Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. *J Bacteriol* 180:136–142.
  26. Chen Y, McClane BA, Fisher DJ, Rood JI, Gupta P. 2005. Construction of an alpha toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. *Appl Environ Microbiol* 71:7542–7547. <https://doi.org/10.1128/AEM.71.11.7542-7547.2005>.
  27. Phillips ZE, Strauch MA. 2002. *Bacillus subtilis* sporulation and stationary phase gene expression. *Cell Mol Life Sci* 59:392–402. <https://doi.org/10.1007/s00018-002-8431-9>.
  28. Ohtani K, Hirakawa H, Paredes-Sabja D, Tashiro K, Kuhara S, Sarker MR, Shimizu T. 2013. Unique regulatory mechanism of sporulation and enterotoxin production in *Clostridium perfringens*. *J Bacteriol* 195:2931–2936. <https://doi.org/10.1128/JB.02152-12>.
  29. Kobir A, Poncet S, Bidnenko V, Delumeau O, Jers C, Zouhir S, Grenha R, Nessler S, Noirot P, Mijakovic I. 2014. Phosphorylation of *Bacillus subtilis* gene regulator AbrB modulates its DNA-binding properties. *Mol Microbiol* 92:1129–1141. <https://doi.org/10.1111/mmi.12617>.
  30. Myers GS, Rasko DA, Cheung JK, Ravel J, Seshadri R, DeBoy RT, Ren Q, Varga J, Awad MM, Brinkac LM, Daugherty SC, Haft DH, Dodson RJ, Madupu R, Nelson WC, Rosovitz MJ, Sullivan SA, Dimitrov HKGI, Watkins KL, Mulligan S, Benton J, Radune D, Fisher DJ, Atkins HS, Hiscox T, Jost BH, Billington SJ, Songer JG, McClane BA, Titball RW, Rood JI, Melville SB, Paulsen IT. 2006. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Res* 16:1031–1040. <https://doi.org/10.1101/gr.5238106>.
  31. Xiao Y, van Hijum SA, Abbe T, Wells-Bennik MH. 2015. Genome-wide transcriptional profiling of *Clostridium perfringens* SM101 during sporulation extends the core of putative sporulation genes and genes determining spore properties and germination characteristics. *PLoS One* 10:e0127036. <https://doi.org/10.1371/journal.pone.0127036>.
  32. Shafikhani SH, Leighton T. 2004. AbrB and Spo0E control the proper timing of sporulation in *Bacillus subtilis*. *Curr Microbiol* 48:262–269. <https://doi.org/10.1007/s00284-003-4186-2>.
  33. Qi M, Mei F, Wang H, Sun M, Wang G, Yu Z, Je Y, Li M. 2015. Function of global regulator CodY in *Bacillus thuringiensis* BMB171 by comparative proteomic analysis. *J Microbiol Biotechnol* 25:152–161. <https://doi.org/10.4014/jmb.1406.06036>.
  34. Strauch MA, Webb V, Spiegelman G, Hoch JA. 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. *Proc Natl Acad Sci U S A* 87:1801–1805. <https://doi.org/10.1073/pnas.87.5.1801>.
  35. Scotcher MC, Rudolph FB, Bennett GN. 2005. Expression of *abrB310* and *sinR*, and effects of decreased *abrB310* expression on the transition from acidogenesis to solventogenesis, in *Clostridium acetobutylicum* ATCC 824. *Appl Environ Microbiol* 71:1987–1995. <https://doi.org/10.1128/AEM.71.4.1987-1995.2005>.
  36. Deguchi A, Miyamoto K, Kuwahara T, Kaneko I, Li J, McClane BA, Akimoto S. 2009. Genetic characterization of type A enterotoxigenic *Clostridium perfringens* strains. *PLoS One* 4:e5598. <https://doi.org/10.1371/journal.pone.0005598>.
  37. Li J, McClane BA. 2014. Contributions of NanI sialidase to Caco-2 cell adherence by *Clostridium perfringens* type A and C strains causing human intestinal disease. *Infect Immun* 82:4620–4630. <https://doi.org/10.1128/IAI.02322-14>.
  38. Li J, McClane BA. 2008. A novel small acid soluble protein variant is important for spore resistance of most *Clostridium perfringens* food poisoning isolates. *PLoS Pathog* 4:e1000056. <https://doi.org/10.1371/journal.ppat.1000056>.
  39. Li J, Chen J, Vidal JE, McClane BA. 2011. The Agr-like quorum-sensing system regulates sporulation and production of enterotoxin and beta2 toxin by *Clostridium perfringens* type A non-food-borne human gastrointestinal disease strain F5603. *Infect Immun* 79:2451–2459. <https://doi.org/10.1128/IAI.00169-11>.
  40. Bannam TL, Rood JI. 1993. *Clostridium perfringens*-*Escherichia coli* shuttle vectors that carry single antibiotic resistance determinants. *Plasmid* 29:223–235.
  41. Li J, McClane BA. 2006. Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. *Appl Environ Microbiol* 72:4561–4568. <https://doi.org/10.1128/AEM.00177-06>.
  42. Melville SB, Labbe R, Sonenshein AL. 1994. Expression from the *Clostridium perfringens* *cpe* promoter in *C. perfringens* and *Bacillus subtilis*. *Infect Immun* 62:5550–5558.