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Unique Regulatory Mechanism of Sporulation and Enterotoxin Production in *Clostridium perfringens*

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***Clostridium perfringens* causes gas gangrene and gastrointestinal (GI) diseases in humans. The most common cause of *C. perfringens*-associated food poisoning is the consumption of *C. perfringens* vegetative cells followed by sporulation and production of enterotoxin in the gut. Despite the importance of spore formation in *C. perfringens* pathogenesis, the details of the regulation of sporulation have not yet been defined fully. In this study, microarray and bioinformatic analyses identified a candidate gene (the RNA regulator *virX*) for the repression of genes encoding positive regulators (Spo0A and sigma factors) of *C. perfringens* sporulation. A *virX* mutant constructed in the food poisoning strain SM101 had a much higher sporulation efficiency than that of the wild type. The transcription of *sigE*, *sigF*, and *sigK* was strongly induced at 2.5 h of culture of the *virX* mutant. Moreover, the transcription of the enterotoxin gene was also strongly induced in the *virX* mutant. Western blotting confirmed that the levels of enterotoxin production were higher in the *virX* mutant than in the wild type. These observations indicated that the higher levels of sporulation and enterotoxin production in the *virX* mutant were specifically due to inactivation of the *virX* gene. Since *virX* homologues were not found in any *Bacillus* species but were present in other clostridial species, our findings identify further differences in the regulation of sporulation between *Bacillus* and certain *Clostridium* species. The *virX* RNA regulator plays a key role in the drastic shift in lifestyle of the anaerobic flesh eater *C. perfringens* between the vegetative state (for gas gangrene) and the sporulating state (for food poisoning).**

Clostridium perfringens is a causative agent of a wide variety of enteric and histotoxic diseases (1, 2). Among five types (A to E), *C. perfringens* type A is known to cause two types of infectious diseases in humans. The first is gas gangrene (clostridial myonecrosis), in which *C. perfringens* typically contaminates a skin wound as a spore from the soil and then rapidly grows and produces various histolytic toxins and enzymes that destroy the surrounding muscles and connective tissues. The second type consists of a few strains (~5% of all *C. perfringens* type A isolates) that have the ability to produce enterotoxin (CPE) (3). In both diseases, spores play an essential role in the pathogenesis of *C. perfringens*.

The CPE-producing strains are responsible annually for nearly 1 million cases of *C. perfringens* food poisoning, which ranks as the second most commonly reported bacterial food-borne disease in the United States (4). When vegetative CPE-producing *C. perfringens* cells grown in foods are ingested, the cells reach the small intestine and begin to sporulate. During the sporulation process, a large amount of CPE is produced and forms pores in the membranes of the intestinal epithelial cells, which results in watery diarrhea.

The sporulation process in *Bacillus* and *Clostridium* is initiated by a wide range of environmental and physiological signals induced by cell density, the Krebs cycle, and nutrient starvation (5). In *Bacillus*, sporulation-specific orphan histidine kinases integrate these signals and initiate a complex phosphorelay that leads to an increased concentration of phosphorylated Spo0A (Spo0A~P), the master regulator of sporulation (6, 7). Spo0A~P directly activates 121 genes, including those required for polar septum formation (8). Once Spo0A becomes an active form (Spo0A~P), sporulation-specific sigma factors (SigF, SigE, SigG, and SigK) are sequentially activated (7, 9), which then activates hundreds of genes, and spores are formed and released from the mother cells (6, 7). In *Clostridium*, many genes related to the sporulation function are missing compared with *Bacillus subtilis*; in particular, the genes for the phosphorelay essential to *B. subtilis* sporulation are totally absent (10). Although the genes from *spo0A* to *sigK* are the same in both genera, recent evidence has suggested that the sporulation-specific RNA polymerase sigma factors are not expressed in a sequential fashion in *Clostridium* as in *Bacillus* species (11–13). Following the early forespore-specific gene *sigF*, the mother cell-specific *sigK* gene becomes expressed at sufficiently low levels to induce expression of the *sigE* gene, which in turn provides feedback and allows transcription of *sigK* (13). Interestingly, the late forespore-specific SigG protein is solely dependent on SigF (12). Sporulation in *C. perfringens* also seems to be dependent on an Agr-like quorum-sensing (QS) system that positively regulates sporulation and CPE production of *C. perfringens* (11).

In a previous study, the *virX* gene was reported to be a regulator for several toxin genes that are expressed at the log phase of vegetative growth (14). The *virX* gene encodes 51 amino acids that form a zinc-finger-containing peptide, and it positively regulates the theta-, alpha-, and kappa-toxin genes (*pfoA*, *plc*, and *colA*). Moreover, it was shown that *virX* acts as a small RNA regulator for

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the control of virulence in *C. perfringens* (14). In this report, we describe that *virX* is also a regulatory RNA gene for massive control of the sporulation-related genes and the enterotoxin gene in food-borne *C. perfringens* strains, which constitutes a unique regulatory system for spore formation that is distinct from that of *Bacillus* species.

MATERIALS AND METHODS

Strains, culture conditions, and plasmids. *C. perfringens* strains 13 (15), TS186/pJIR418, and TS186/pTS907 (14) were cultured in GAM broth (Nissui, Japan) at 37°C under anaerobic conditions as described previously (16). The CPE-positive *C. perfringens* strain SM101 (17) and its derivative strains were cultured in fluid thioglycolate (FTG, Difco, BD) or TGY (3% tryptic soy broth [Becton Dickinson], 2% glucose, 1% yeast extract, and 0.1% cysteine). *Escherichia coli* strain DH5α and plasmid pUC118 were used for general cloning procedures. Plasmid pJIR418 (18) was used as an *E. coli*-*C. perfringens* shuttle vector and was transformed by electroporation-mediated transformation as previously described (16, 17).

Construction of a *virX* mutant strain. A DNA fragment containing the *virX* region of SM101 (19) was amplified by a PCR using primer 1 (5′-TGGCCGGTCCTAAAGCTGTATCCA-3′) and primer 4 (5′-TGCCT TCATCAACTAATTGCT-3′). The amplified fragment was inserted into the HincII site of pUC118, which was used as a template for an inverse PCR using primers *virX*-null-2 (5′-TCTGACGCTTAAAATAAGCTAG T-3′) and *virX*-null-3 (5′-TAGAGCAATCTAACTTATGATT-3′). The erythromycin resistance gene (*ermBP*) was then cloned into the space between primers *virX*-null-2 and *virX*-null-3. The resultant plasmid was transformed into wild-type SM101. The transformants resulting from homologous recombination were screened on a brain heart infusion (BHI) plate containing erythromycin (25 μg/ml). Allelic-exchange mutation of the *virX* gene due to a double-crossover recombination was confirmed by PCR (see Fig. S1 in the supplemental material).

Construction of a *virX*-complemented strain. Single-copy complementation of the *virX* mutant with wild-type and mutated *virX* genes was performed by single-crossover insertion of *virX* derivatives into the chromosome, using the *plc* gene (encoding alpha-toxin) for the insertion locus. First, the chloramphenicol resistance gene (*catP*) was amplified from pJIR418 by PCR, and the fragment was inserted into the HincII site of pUC118 (pCM118). The PCR-amplified *plc* fragment was then inserted into the HindIII site of pCM118 (pKO11). Finally, the intact *virX* fragment was amplified and inserted into the SmaI site of pKO11 (pKO12). The resulting plasmids, pKO11 and pKO12, were transformed into SM101 (wild type) and KO101 (*virX* mutant), respectively, by electroporation. The transformants resulting from single-crossover homologous recombination were screened on a BHI plate containing both erythromycin (25 μg/ml) and chloramphenicol (25 μg/ml). Using the same method, we also constructed KO101 derivatives that were complemented with mutated *virX* genes: KO103 and KO104. KO103 and KO104 were complemented with *virX* genes mutated at the 4th codon (AAA for Lys) to a stop codon (TAA) and at the 42nd codon (AAA for Lys) to a stop codon, derived from pTS913 and pTS915, respectively, in a previous study (14). All single-copy complementations of *virX* inserted into the chromosomal *plc* locus were confirmed by PCR (see Fig. S1 in the supplemental material).

Sporulation assay. The wild-type strain (SM101), the *virX* mutant strain (KO101), and the complemented strains (KO102, KO103, and KO104) were inoculated into FTG medium, and the inoculated tubes were heat shocked at 70°C for 20 min. The strains were cultured at 37°C for 24 h, and 0.1 ml of each culture was inoculated and cultured into a second 10 ml of FTG medium at 37°C for 15 h. Four milliliters of the second precultured strain was inoculated into 200 ml of Duncan-Strong (DS) sporulating medium (1.5% proteose peptone [Becton Dickinson], 1% sodium phosphate, 0.4% soluble starch, 0.4% yeast extract, 0.1% sodium thioglycolate). Spores were examined at 6 or 24 h of culture. To count the number of heat-resistant spores, the 24-h DS-grown cultures were heated at 70°C for 20 min, serially diluted with phosphate-buffered saline (PBS), plated onto BHI agar, and incubated anaerobically at 37°C for 18 to 24 h, and then the colonies were counted.

Northern blot hybridizations. Total RNA from *C. perfringens* vegetative cells was extracted according to a previously described method (14). From sporulating cultures in DS medium, RNA was isolated by a modified method. Briefly, cell pellets from sporulating cultures were suspended with solution A without SDS (20 mM sodium acetate, pH 5.0, 1 mM EDTA, pH 8.0), and then sodium acetate-saturated phenol (pH 5.0) was added. Zirconia beads were added to the cell suspension, and the tubes were vigorously shaken with a Beads crusher (Taitec, Japan) for 5 min. After removal of the beads, the cells were centrifuged, and a routine RNA extraction method was applied. Northern hybridization and signal detection were done with an AlkPhos-Direct kit (GE Healthcare).

Western blotting. To examine the production of CPE, the *C. perfringens* strains were cultured by the same method as that used for the sporulation analysis (see above). Cells were collected from 8-h cultures and washed with PBS twice. The cells were then resuspended with PBS and disrupted by sonication until more than 95% of the cells were lysed. The lysed cells were centrifuged, and the supernatant was recovered. The protein concentration of the supernatant was measured by the Bradford protein assay method, and 1 μg of protein was used for Western blotting using rabbit anti-CPE antiserum. ECL Plus Western blotting detection reagent (GE Healthcare) was used for detection of immunoreactive bands.

Analysis of morphology. Samples (0.5 ml) of 6-h cultures of *C. perfringens* strains in DS medium were centrifuged, and the cells were collected. The cells were washed with PBS twice and then resuspended with 15 μl of PBS. A final concentration of 0.1 μg/μl of FM4-64 was added to the cells and mixed. The cells were incubated at room temperature for 10 min and then subjected to phase-contrast and fluorescence microscopic analyses using a Biozero 8000 microscope (Keyence, Japan).

Microarray assays. Microarray experiments using wild-type strain 13 and the *virX* mutant TS186 (14) were performed according to previously described methods (20).

Microarray data accession number. The microarray data have been deposited in NCBI's Gene Expression Omnibus under GEO Series accession number GSE37998.

RESULTS

***virX* negatively regulates sporulation-specific sigma factors in *C. perfringens* under vegetative and sporulation conditions.** Our previous study indicated that the regulatory RNA *virX* positively regulates transcription of alpha-toxin (*plc*), kappa-toxin (*colA*), and theta-toxin (*pfoA*) (14). In an attempt to identify further genes that are regulated by *virX* in the *C. perfringens* strain 13 genome (21), we used a custom DNA microarray that contained PCR-generated strain 13 DNA (20) and compared the transcriptional levels of each chromosomal gene between wild-type strain 13 and the $virX_{strain13}$ mutant strain TS186 (14) in 3-h nutrient-rich GAM cultures. The $virX_{strain13}$ gene appeared to control the transcription of 53 and 110 genes, positively (see Table S1 in the supplemental material) and negatively (Table S2), respectively, at 3 h of growth of strain 13. Strikingly, we found that the genes encoding all four sporulation-related RNA polymerase σ factors (*sigF*, *sigE*/*sigG*, and *sigK*) were upregulated in TS186 (Table 1). To confirm these results, we performed Northern analyses on total RNAs isolated from 4-h GAM cultures. Indeed, the results showed that transcription of all four sporulation-specific σ factors was upregulated in TS186 (Fig. 1A). Note that *sigE* and *sigG* are part of the *spoIIGA*-*sigE*-*sigG* operon (12), and therefore the *sigE* and *sigG* transcript can be detected with a *sigG* probe. The upregulation of σ factors in TS186 decreased to wild-type levels upon complementation with the wild-type $virX_{strain13}$ gene (Fig. 1A). Collec-

TABLE 1 Sporulation-related genes negatively regulated by *virX*

CPE no.	Gene	Product	Fold change ($\log_2$)	*t* test *P* value
CPE1753	*spoIVA*	Stage IV sporulation protein A	3.02	8.74E−04
CPE1761	*sigG*	RNA polymerase sigma G factor	2.00	1.06E−04
CPE1762	*sigE*	RNA polymerase sigma E factor	3.28	2.64E−04
CPE1763	*spoIIGA*	Sporulation protein SpoIIGA	2.98	1.16E−03
CPE1812	*spo0A*	Transcription factor Spo0A	2.23	1.12E−03
CPE2048	*sigF*	RNA polymerase sigma F factor	4.53	6.72E−04
CPE2049	*spoIIAB*	Anti-sigma F factor antagonist	4.61	1.29E−04
CPE2050	*spoIIAA*	Anti-sigma F factor antagonist	4.41	5.62E−05
CPE2473	*spoIIE*	Stage II sporulation protein E	5.40	2.06E−04

tively, these results suggest that $virX_{strain13}$ negatively regulates all four sporulation-specific σ factors during vegetative growth of *C. perfringens* strain 13.

Since a previous report (21) indicated that *C. perfringens* strain 13 has a frameshift mutation in the gene of the master regulator, *spo0A* (22, 23), we examined the ability of spore formation of strain 13 under the same conditions as those used for SM101. As expected, strain 13 did not form spores (data not shown). Therefore, we newly constructed a *virX*-knockout mutant (KO101) and a complemented strain (KO102) from the sporulation-proficient food poisoning strain SM101 to evaluate whether *virX* plays a role in the sporulation of *C. perfringens*. These strains were cultured in DS medium, and they all showed similar growth curves, which could be divided into early-log (~2.5 h), mid- to late-log (~6 h), and stationary (~7 h) phases.

Northern analysis of the master regulator of sporulation, *spo0A*, showed that *virX* seemed to have no regulatory effect on *spo0A* transcription during the sporulation process of SM101 (Fig. 1B). Much as in *B. subtilis*, σ^F was the earliest and most sporulation-specific σ factor to be expressed (12, 24). In wild-type SM101, *sigF* was expressed after 2.5 h of initiation of sporulation as a 2.6-kb *sigF* transcript and at very low levels (Fig. 1B). Interestingly, an ~1.0-kb transcript that also reacted with the *sigF* probe appeared after 2.5 h of sporulation, which indicates that *sigF* is transcribed from two promoters, giving 2.6-kb *spoIIAA-spoIIAB-sigF* and ~1.0-kb *sigF* transcripts (Fig. 1B). For the $virX_{SM101}$ mutant KO101, strong expression of both the 2.6- and ~1.0-kb transcripts was observed in a 2.5-h DS culture (Fig. 1B) but decreased to levels lower than those in SM101 after 3.5 h (Fig. 1B). The negative regulatory effect of *virX* was more pronounced on the small *sigF* transcript (~1.0 kb), with significantly more overexpression of *sigF* during early sporulation and lower *sigF* levels than at later stages compared with the large (2.6 kb) *spoIIAA-spoIIAB-sigF* transcript (Fig. 1B).

In *C. perfringens*, the low levels of transcription of *sigK* required for *sigE* activation precede *sigE* transcription, which in turn activates *sigK* at a later stage of sporulation (13). In this study, no early (i.e., 1.5 and 2.5 h of DS culture) transcription of *sigK* was detected by Northern analysis of wild-type SM101, and this transcription was detectable only after 3.5 h (Fig. 1B). In contrast, the earliest mother cell-specific σ factor is σ^E, which was expressed as a 3.5-kb *spoIIGA-sigE-sigG* transcript after 2.5 h of sporulation, making the ~1.1-kb transcript encoding only *sigG* the most predominant at later stages of sporulation (i.e., 4.5 and 5.5 h) (Fig. 1B). A detectable level of a 1.0-kb *sigK* transcript was present in wild-type SM101 after only 3.5 h of sporulation (Fig. 1B).

More strikingly, in the *virX* mutant KO101, 3.5-, 1.1-, and 1.0-kb transcripts, specific for *spoIIGA-sigE-sigG*, *sigG*, and *sigK*, respectively, were upregulated during early (2.5 h) sporulation. We also assayed the transcription levels of *virX* during sporula-

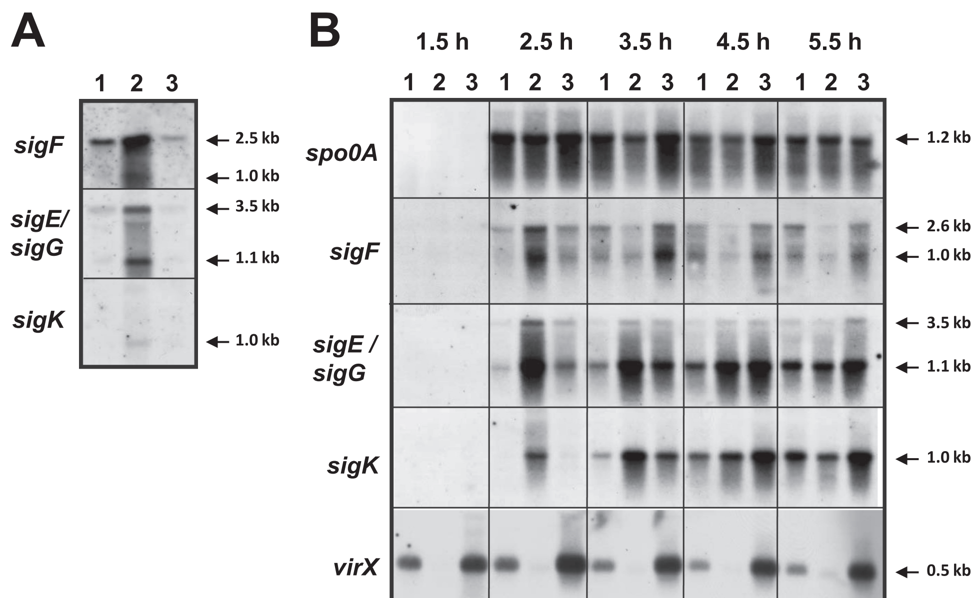

FIG 1 Effects of a *virX* mutation on transcription of sporulation-specific sigma factors of *C. perfringens* strains. (A) The levels of *sigF*, *sigE*/*sigG*, and *sigK* transcripts in total RNAs isolated from 4-h nonsporulating GAM cultures were analyzed by Northern blotting. Lanes: 1, strain 13(pJIR418) (wild type); 2, TS186(pJIR418) ($virX_{strain13}$ mutant); 3, TS186(pTS907) (TS186 complemented with wild-type $virX_{strain13}$). (B) Northern blot results are shown for the transcription of *spo0A*, *sigF*, *sigE*/*sigG*, *sigK*, and *virX* during early logarithmic growth (1.5 and 2.5 h) and logarithmic growth (3.5, 4.5, and 5.5 h) of DS sporulation cultures. Lanes: 1, SM101 (wild type); 2, KO101 ($virX_{SM101}$ mutant); 3, KO102 (KO101 complemented with wild-type $virX_{SM101}$).

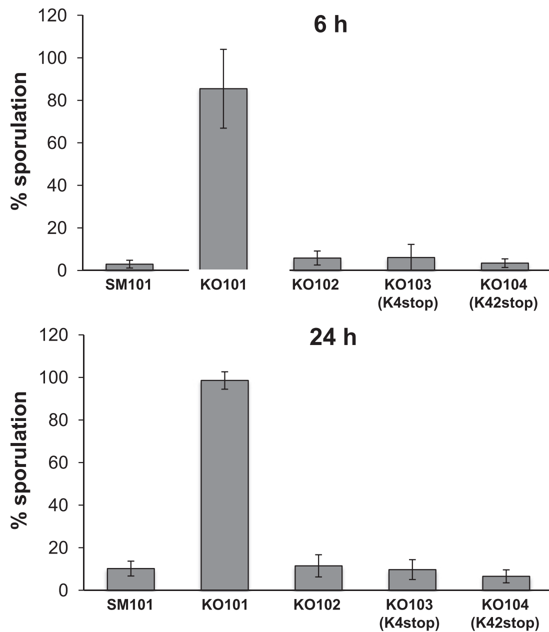

FIG 2 Sporulation efficiencies of various *C. perfringens* strains. To measure sporulation efficiency, 6- and 24-h sporulating cultures of SM101 (wild type), KO101 (*virX* mutant), KO102 (complemented with wild-type *virX*), KO103 (complemented with *virX*-K4stop), and KO104 (complemented with *virX*-K42stop) were directly plated onto BHI agar to determine the total numbers of CFU. The cultures were then heat treated (70°C, 20 min) and plated onto BHI agar to determine the numbers of CFU from heat-resistant spores. Both were incubated overnight under anaerobic conditions at 37°C. Sporulation efficiency (%) was calculated as follows: (heat-resistant CFU ml^{-1}/total CFU ml^{-1}) $\times$ 100. The data are presented as means ± standard deviations (SD) calculated from at least 3 independent experiments.

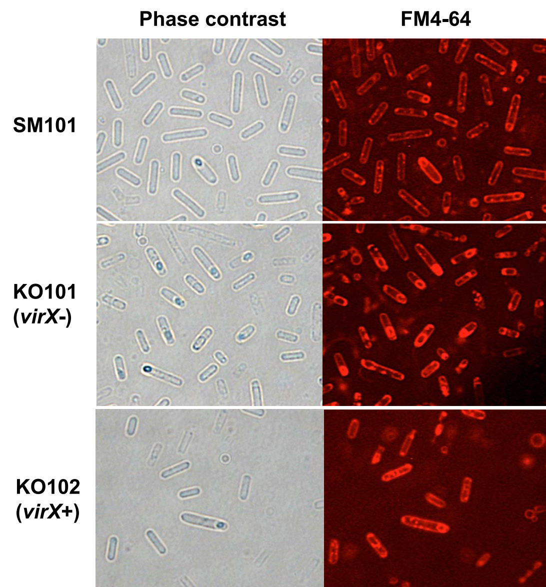

FIG 3 Cell morphologies of *C. perfringens* SM101 and its derivatives grown under sporulation conditions. Morphologies of 6-h DS sporulation cultures of *C. perfringens* SM101 (wild type), KO101 (*virX* mutant), and KO102 (KO101 complemented with wild-type *virX*) were analyzed by phase-contrast microscopy and fluorescence microscopy with FM4-64.

tion, and the results showed that *virX* transcription decreased only slightly during the progression of sporulation in SM101 (Fig. 1B), indicating that *virX* transcription remained active throughout the sporulation process. Importantly, the effect of the absence of *virX* in KO101 on the transcription of all four sporulation-specific σ factors was restored to wild-type levels in the complemented strain KO102 (Fig. 1B). Collectively, these results indicate that (i) *virX* is well transcribed during the *C. perfringens* sporulation process and (ii) *virX* negatively regulates the expression of *sigF*, *sigE*, *sigK*, and *sigG* in wild-type *C. perfringens*.

***virX* mutation increases the sporulation efficiency of *C. perfringens*.** To quantify the effect of a *virX* mutation on the sporulation efficiency of *C. perfringens*, 6- and 24-h DS cultures were assayed for heat-resistant CFU. Interestingly, 6-h cultures of the $virX_{SM101}$ mutant KO101 had ~28-fold more heat-resistant CFU than the SM101 cultures (Fig. 2). After 24 h, the KO101 cultures had nearly 10-fold more heat-resistant CFU than the SM101 cultures, and they reached a sporulation efficiency of ~95% (Fig. 2), indicating that sporulating cultures of KO101 not only sporulate with a high efficiency but also begin to sporulate earlier than SM101 spores. Moreover, complementation with intact *virX* (KO102) resulted in a drastic decrease of heat-resistant spores, almost to the same levels as those of SM101, at both 6 and 24 h (Fig. 2), which clearly indicates that *virX* is a strong repressor of *C. perfringens* sporulation.

Since *virX* has been reported to act as an RNA regulator on toxin genes (14), we also made complemented strains of KO101 with *virX* genes that had a nonsense (stop codon) mutation at the codon for K4 (K4stop) or K42 (K42stop) in the protein coding region of *virX*. The sporulation efficiencies of the resultant complemented strains (KO103 and KO104) had no significant difference from that of KO102 (Fig. 2). These data indicate that *virX* also act as a RNA regulator but not as a regulatory protein/peptide and that the RNA molecule negatively controls sporulation in *C. perfringens*, in just the same manner as that of toxin regulation in strain 13 (14).

We further evaluated whether the absence of *virX* would affect the morphology of sporulating cells grown in DS medium by using phase-contrast microscopy. *C. perfringens* wild-type SM101 grown for 6 h in DS medium showed some cells harboring refractive polar prespores (Fig. 3). By fluorescence microscopy analysis using the fluorescent dye FM4-64 (specific for membrane lipids), it was possible to delimit the membrane of the polar prespore in SM101 (Fig. 3). Similarly, for sporulating KO101 cells, the prespore and mother cell compartments were clearly separated by a membrane visible by FM4-64 staining (Fig. 3), indicating that the $virX_{SM101}$ mutant KO101 has an asymmetrical sporangium phenotype similar to that of wild-type cells under sporulation conditions. Complementation of KO101 with wild-type *virX* (KO102) had no effect on the FM4-64-staining cell morphology compared to that of SM101 and KO101 (Fig. 3). These results indicate that *virX* is not involved in cell morphology during sporulation of *C. perfringens* cells.

***virX* represses *cpe* transcription during sporulation of *C. perfringens*.** During the sporulation of *C. perfringens*, the expression of the enterotoxin gene (*cpe*) is under the direct control of sporulation-specific σ factors (σ^E and σ^K) that are expressed in the mother cell compartment of the sporulating cell (12, 13, 17, 25). Since *virX* represses σ^E and σ^K during early sporulation, we tested the hypothesis that *virX* would also repress the expression and

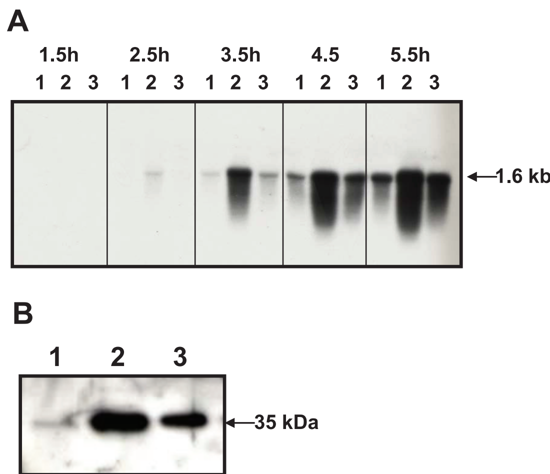

FIG 4 Effects of *virX* mutation on transcription and production of *C. perfringens* enterotoxin. (A) Total RNA was extracted at various times from DS sporulation cultures and analyzed by Northern blotting. Lanes: 1, SM101 (wild type); 2, KO101 (*virX* mutant); 3, KO102 (KO101 complemented with wild-type *virX*). (B) Eight-hour DS sporulation cultures of *C. perfringens* strains were analyzed by Western blotting using rabbit CPE antiserum. Lanes: 1, SM101 (wild type); 2, KO101 (*virX* mutant); 3, KO102 (KO101 complemented with wild-type *virX*).

production of CPE. Northern analyses of SM101 DS cultures showed that the transcription of *cpe* began after 3.5 h of sporulation in SM101 (Fig. 4A), while transcription of *cpe* in the *virX* mutant KO101 began ~1 h earlier than that in SM101 (Fig. 4A). At later stages of sporulation, transcriptional levels of *cpe* remained higher in KO101 than in SM101 (Fig. 4A). Western blot analyses showed that CPE levels in 8-h sporulating cultures of KO101 were approximately 6 times higher than those in SM101 (Fig. 4B). The complemented strain KO102 restored the levels of *cpe* transcript and CPE toxin to nearly wild-type levels (Fig. 4A and B). These results indicate that *virX* negatively regulates the transcription and production of CPE during sporulation of *C. perfringens*.

DISCUSSION

C. perfringens possesses great toxin versatility and is capable of producing up to 18 different toxins (3, 26, 27). The majority of these toxins are under the control of regulatory mechanisms exclusive to the vegetative growth phase (16, 20, 28). Recent evidence has demonstrated that alpha- and theta-toxin production is also dependent on an Agr-like quorum-sensing system that plays roles in toxin regulation under vegetative growth (29, 30) and is also required for efficient sporulation and production of CPE (11). Our results demonstrate not only that the regulatory RNA *virX* is required for positive regulation of virulence factors (alpha-, kappa-, and theta-toxin) during vegetative growth (14) but, in contrast to the Agr-like quorum-sensing system, that *virX* RNA acts as a repressor of sporulation in *C. perfringens*. Here it was shown that the transcription levels of *spo0A* were not affected in the *virX* mutant KO101, indicating that the negative effect of *virX* on sporulation does not occur at the level of *spo0A*. Instead, it is likely that *virX* negatively regulates sporulation by repressing transcription of the sporulation-associated and forespore-specific σ factor σ^{F}. Recent studies have shown that σ^{F} regulates expression of the remaining three sporulation-associated σ factors (σ^{E}, σ^{K}, and σ^{G}) (12, 13). Indeed, in a *C. perfringens sigF*-knockout mutant, there was no production of σ^{E}, σ^{K}, or σ^{G} (12), supporting our hypothesis that *virX* negatively regulates the transcription of all four sporulation-specific σ factors, likely through σ^{F}.

The expression of *cpe* during *C. perfringens* sporulation is under the direct control of the mother cell-specific σ factors σ^{E} and σ^{K}, since the promoter region of *cpe* has one σ^{K}-dependent promoter and two σ^{E}-dependent promoters (17). Given the cross-regulation between σ^{K} and σ^{E} and the fact that both are directly controlled by σ^{F}, our results also suggest that *virX* negatively regulates the transcription of *cpe* and production of CPE through the forespore-specific σ factor σ^{F}. It was notable that unlike in the previous study indicating that expression of *sigK* precedes expression of *sigE* (13), we were unable to detect *sigK* transcription by Northern blotting at 2.5 h of sporulation. A likely explanation is that the amount of early *sigK* transcript being produced under our sporulating conditions was much lower than the detection limit of Northern blot analyses. Indeed, we checked the expression of *sigK* by quantitative reverse transcription-PCR (qRT-PCR), and small amounts of the *sigK* transcript (5 to 15% of the *sigF* mRNA level) were detected in sporulating cultures at 2.5 h (data not shown). Overall, the reduction of transcription levels of *sigF*, *sigE*, and *sigG* during late sporulation in the $virX_{SM101}$ mutant KO101 may have been due to an overall decrease of the *sigF* transcript.

Sporulating cells have unique morphological properties, primarily asymmetrical septum formation, with the formation of a large progeny called the mother cell and a small progeny termed the forespore. In this study, construction of a *C. perfringens* SM101 isogenic *virX*-knockout strain did not have any effect on the sporulating cell morphotype. The lack of a role in the sporulating cell morphotype supports the hypothesis that *virX* acts uniquely on the earliest forespore sporulation-associated σ factor, σ^{F}. *virX* mutation also led to a striking increase in heat-resistant spores at a very early stage of sporulation, indicating that a quick upregulation of the sporulation-specific σ factor cascade leads to rapid spore formation, a phenomenon that might have certain implications in pathogenesis, since food poisoning strains with low levels of *virX* might be more prone to sporulate under food poisoning conditions. Further comparative studies on the expression of *virX* in *C. perfringens* food poisoning versus non-food-borne isolates should clarify this point.

Our previous report indicated that *virX* is a positive regulator of several toxin genes in *C. perfringens* strain 13 (the gas gangrene strain) (14). In contrast, this study suggested that *virX* is also a negative regulator of spore formation in SM101 (a food poisoning strain). These results suggest that *virX* strongly represses spore formation under nutrient-rich conditions (i.e., in the human body), which leads to the VirR–VirS–VR-RNA-mediated expression of various toxins and enzymes (20) that will degrade environmental nutritional sources in order to obtain multiple nutrition for the organism's growth and survival. In contrast, under sporulation-favoring conditions, the repression of the sporulation-related sigma factors by *virX* would be loosened up through unknown environmental signals that allow the cells to start sporulation for survival in the environment. The *virX* RNA regulator would play a key role in the drastic shift in lifestyle of the anaerobic flesh eater *C. perfringens* in response to various environmental conditions. Most importantly, the elucidation of the signals and/or conditions that affect the regulatory function of *virX* is a prerequisite to fully understanding the mechanism of spore for-

mation in *C. perfringens*, which could lead to many applications for prevention of *C. perfringens* infection and food poisoning.

Lastly, as shown in Table S3 in the supplemental material, *virX* homologues (based on amino acid sequences) could not be found in any *Bacillus* species, *Clostridium tetani*, or *Clostridium difficile*, while there are multiple copies of *virX* homologues in limited species of *Firmicutes*, including *Clostridium botulinum*, *Clostridium acetobutyricum*, and *C. perfringens* (although *C. perfringens* has four *virX* homologues, only the *virX* studied here has regulatory RNA activity for toxin production and sporulation [data not shown]). This highly biased existence of *virX* homologues among these genera might correspond to the complexity of the world of various spore formers and could determine their lifestyles on earth.

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