Complementation of a *Clostridium perfringens* *spo0A* Mutant with Wild-Type *spo0A* from Other *Clostridium* Species

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To evaluate whether *C. perfringens* can be used as a model organism for studying the sporulation process in other clostridia, *C. perfringens* *spo0A* mutant IH101 was complemented with wild-type *spo0A* from four different *Clostridium* species. Wild-type *spo0A* from *C. acetobutylicum* or *C. tetani*, but not from *C. botulinum* or *C. difficile*, restored sporulation and enterotoxin production in IH101. The ability of *spo0A* from *C. botulinum* or *C. difficile* to complement the lack of spore formation in IH101 might be due, at least in part, to the low levels of *spo0A* transcription and Spo0A production.

Under environmental stress, *Bacillus* and *Clostridium* species undergo asymmetric cell division, or sporulation (13). The initiation of sporulation in *Bacillus subtilis* is controlled by the phosphorylation state of the sporulation transcription factor, Spo0A (5). As a member of the response regulator, Spo0A orchestrates changes in gene transcription during the transition from growth to sporulation (13). Counterparts of *B. subtilis* Spo0A have been detected in many other *Bacillus* and *Clostridium* species (3, 4, 8, 16), and recent studies have presented evidence that (i) *Clostridium acetobutylicum* Spo0A transcriptionally activates genes for sporulation and solvent formation (8, 9, 14) and (ii) expression of *spo0A* is essential for the formation of endospores and the production of *Clostridium perfringens* enterotoxin (CPE) by *C. perfringens* type A (10).

Although the processes of sporulation in *Bacillus* and *Clostridium* species are assumed to be similar, recent genome sequencing has identified some important differences. One example of the difference is that the phosphorelay pathway required to activate Spo0A in *Bacillus* species is absent in *Clostridium* species (4, 8, 16, 17). To gain insight into the fundamental differences between the sporulation mechanisms of *Clostridium* and *Bacillus* species, it is essential to perform molecular analyses of sporulation-specific genes in clostridia. Research on many pathogenic *Clostridium* species, such as *C. botulinum*, *C. difficile*, and *C. tetani*, is hampered by the lack of genetic tools to introduce knockout mutations into the genome. However, mutagenesis is possible in *C. perfringens*. Therefore, we used *C. perfringens* as a model organism with which to study the sporulation process in other pathogenic clostridia. We hypothesized that if the mechanism of Spo0A-regulated sporulation in *C. perfringens* is similar to that in other *Clostridium* species, then the lack of endospore formation in a *spo0A* knockout mutant of *C. perfringens* should be complemented by wild-type *spo0A* from other clostridia. To evaluate this hypothesis, in this study we (i) constructed complementing plasmids carrying wild-type *spo0A* from *C. acetobutylicum*, *C.*

botulinum, *C. difficile*, or *C. tetani*, (ii) introduced these complementing plasmids into a *C. perfringens* *spo0A* knockout mutant, and (iii) evaluated the sporulation capabilities of the complemented strains.

The wild-type *spo0A* open reading frame (ORF) plus a ~400-bp upstream sequence of *C. botulinum* (strain Hall), *C. difficile* (strain 630), and *C. tetani* (CN655) were PCR amplified from each genomic DNA using primer pairs CPP61–CPP62, CPP51–CPP52, and CPP59–CPP60, respectively (Table 1). The *spo0A* ORF and ~400-bp upstream region of *C. acetobutylicum* were PCR amplified from pMSPOA (Table 2) using primers CPP70 and CPP71 (Table 1). The amplified PCR products from *C. acetobutylicum*, *C. botulinum*, *C. difficile*, and *C. tetani* were then cloned into pCR-XL-TOPO (Invitrogen) to create pIH1, pIH3, pIH8, and pIH13, respectively (Table 2). The DNA inserts in these plasmids were sequenced to ensure that no mutation was introduced during PCR amplification. KpnI/XhoI fragments of pIH1, pIH3, pIH8, and pIH13 were then recloned into the KpnI/SalI sites of the *Escherichia coli*–*C. perfringens* shuttle vector pJIR751 (2) to create complementing plasmids pIH2, pIH4, pIH9, and pIH14, respectively (Table 2). The complementing plasmids were then introduced into the *C. perfringens* *spo0A* mutant IH101 by electroporation (7). PCR analyses with *spo0A*-specific internal primers (Table 1) from each *Clostridium* species confirmed the presence of wild-type *spo0A* in IH101 transformants (data not shown).

Having obtained evidence for successful construction of complemented strains, we compared the sporulation capabilities of the complemented strains to that of an *spo0A* mutant as previously described (10). As expected, IH101 exhibited significantly decreased production of heat-resistant spores compared to wild-type SM101 (Table 3) (10), and this lack of ability of the *spo0A* mutant to form heat-resistant spores could be complemented with recombinant plasmid pMRS123, carrying wild-type *spo0A* from *C. perfringens*. When similar experiments were performed with our newly constructed complemented strains, IH101 complemented with the recombinant plasmid pIH14 produced spores at a level similar to that of wild-type SM101 (Table 3). A lower level of heat-resistant spore formation was also observed with IH101 complemented with the recombinant plasmid pIH2 (Table 3). However, a

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TABLE 1. Primers used in this study

Primer name	Primer sequence (5' to 3')	Position ^a	Region(s) amplified
CPP29 CPP96	GAGTGGATGTTAAAAGATGCA ATTCCTTCATATGTTTCTCTCC	−207 +7	<i>C. perfringens</i> <i>spo0A</i> promoter region
CPP51 CPP52	CAGAGCTAGGTATAAGTGGT CCCTAGTGGTTATACCGTTT	−498 +984	<i>C. difficile</i> <i>spo0A</i> plus ~400-bp upstream region
CPP59 CPP60	GGTGAATAGTTCAAGGAATG AAACCATTGGTACGCCTACT	−316 +857	<i>C. tetani</i> <i>spo0A</i> plus ~400-bp upstream region
CPP61 CPP62	GGATGCAGGCATATTAGA AGCCAATGTACAAAAGGC	−457 +950	<i>C. botulinum</i> <i>spo0A</i> plus ~400-bp upstream region
CPP68 CPP69	CAGGAATTGCAAAGGATGGATTGGAAGC GGCATGTATTTGTCCTCTTCCCCAAG	+100 +719	<i>C. perfringens</i> <i>spo0A</i> internal fragment
CPP70 CPP71	GGAGTTTATATTGAATGGATCCTTAAAAAG TTACTATTCCCTGGTGATCATTAAAGAAA	−406 +1119	<i>C. acetobutylicum</i> <i>spo0A</i> plus ~400-bp upstream region
CPP72 CPP73	GGCATAGCTAAGGATGGAATTGAAGC CCTCTACTCCATGCAACCTCTATTGC	+185 +772	<i>C. difficile</i> <i>spo0A</i> internal fragment
CPP74 CPP75	GGTATTGGAAAAGATGGAGTAGAGGC GCTCTTCAACTCTACTAGCGGTGG	+120 +677	<i>C. tetani</i> <i>spo0A</i> internal fragment
CPP76 CPP77	GGTATAGCTAATGATGGAGTAGAGGC GTCTCGACTTGACCTCTTGACCAAG	+102 +710	<i>C. botulinum</i> <i>spo0A</i> internal fragment
CPP93 CPP62	GGAGAGTAGTTCATATGG AGCCAATGTACAAAAGGC	−15 +950	<i>C. botulinum</i> <i>spo0A</i> ORF with NdeI site at ATG
CPP94 CPP52	TGTTTTTCATATGGGGGGA CCCTAGTGGTTATACCGTTT	−9 +984	<i>C. difficile</i> <i>spo0A</i> ORF with NdeI site at ATG
CPP102 CPP103	GAAGAGAAAGAGGCAAGCTTTGCAG GTTTCAACCTGTCCACGTGACCAT	+421 +735	<i>C. acetobutylicum</i> <i>spo0A</i> internal fragment
CPP182 CPP183	CCATATGGGATACATGAAATAGGAGTAC CTTGTAATACGCATTTATAGCCAATGCC	+498 +884	<i>C. difficile</i> <i>spo0A</i> N-terminal domain
CPP184 CPP185	CCATATGGGATACATGAAATAGGCCGT GCACTTAAGCCAATGTACAAAAGGCA	+498 +957	<i>C. botulinum</i> <i>spo0A</i> N-terminal domain
CPP186 CPP193	TGGTTAATAAACCTGAAGTAGGATATG CCATATGGAGTTATTTCAACCTCTAA	−244 +500	<i>C. perfringens</i> <i>spo0A</i> C-terminal domain plus promoter region

^a The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective *spo0A* gene sequence.

recombinant plasmid carrying wild-type *spo0A* either from *C. botulinum* (pIH4) or from *C. difficile* (pIH9) did not complement the mutation in IH101 (Table 3). Collectively, these results indicated that *spo0A* from *C. acetobutylicum* or *C. tetani*, but not from *C. botulinum* or *C. difficile*, was able to complement the sporulation defects of the *C. perfringens* *spo0A* mutant IH101.

Reverse transcription-PCR (RT-PCR) analyses were then performed to investigate whether the inability of *spo0A* from *C. botulinum* or *C. difficile* to restore spore formation in IH101 was a function of the lack of *spo0A* expression. Total RNA was isolated from Duncan-Strong (DS) cultures of *C. perfringens* strains, and *spo0A*-specific mRNA was detected using *spo0A*-specific primers (Table 1) as previously described (7, 10). A *spo0A*-specific RT-PCR product was obtained from RNA of IH101(pIH9), indicating that *C. difficile* *spo0A* can be expressed in *C. perfringens*. However, no *spo0A*-specific RT-PCR

product was obtained from RNA of IH101(pIH4), suggesting that *C. botulinum* *spo0A* was not transcribed in *C. perfringens*.

Next, we examined whether differences in the promoter region were responsible for the lack of *C. botulinum* *spo0A* expression. We constructed a recombinant plasmid, pIH7, carrying *C. botulinum* *spo0A* fused with the *spo0A* promoter region from *C. perfringens* (Table 2). As a control, pIH12, which carries *C. difficile* *spo0A* fused with the *C. perfringens* *spo0A* promoter, was also constructed (Table 2). In addition, since a recent study (18) showed that a chimeric Spo0A, containing the response regulator domain of *B. subtilis* Spo0A fused with the DNA-binding domain of *C. botulinum* Spo0A, could partially restore spore formation in a *B. subtilis* *spo0A* mutant, we constructed similar chimeric plasmids: plasmids pIH19 and pIH21, carrying the *C. perfringens* Spo0A response regulator domain fused to the DNA-binding domain of Spo0A from *C. botulinum* and *C. difficile*, respectively (Table 2). Nucleotide

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<i>C. perfringens</i> strains		
SM101	Electroporatable derivative of a food poisoning type A isolate, NCTC8798	19
IH101	<i>spo0A</i> knockout mutant derivative of SM101	10
IH101(pMRS123)	IH101 complemented with wild-type <i>C. perfringens spo0A</i>	10
Plasmids		
pJIR751	<i>C. perfringens</i> - <i>E. coli</i> shuttle vector; Em ^r	2
pMSPOA	<i>C. acetobutylicum spo0A</i> and its natural promoter inserted into pIMP1	1
pIH1	A 1,527-bp DNA fragment containing the <i>C. acetobutylicum spo0A</i> ORF and a ~400-bp upstream region was cloned into pCR-XL-TOPO	This study
pIH2	A ~1.6-kb KpnI/XhoI fragment from pIH1 was cloned into KpnI/SalI sites of pJIR751	This study
pIH3	A 1,407-bp DNA fragment containing the <i>C. botulinum spo0A</i> ORF and a ~400-bp upstream region was cloned into pCR-XL-TOPO	This study
pIH4	A ~1.5-kb KpnI/XhoI fragment from pIH3 was cloned into KpnI/SalI sites of pJIR751	This study
pIHOAPRO	A 204-bp DNA fragment containing the <i>C. perfringens spo0A</i> upstream region was cloned into pCR-TOPO-XL	This study
pIH5	A 964-bp DNA fragment containing the <i>C. botulinum spo0A</i> ORF with an NdeI site inserted just before ATG was cloned into pCR-XL-TOPO	This study
pIH6	A ~1.0-kb NdeI/PstI fragment from pIH5 was cloned into pIHOAPRO	This study
pIH7	A ~1.2-kb KpnI/XhoI fragment from pIH6 was cloned into KpnI/SalI sites of pJIR751	This study
pIH8	A 1,285-bp DNA fragment containing the <i>C. difficile spo0A</i> ORF and a ~400-bp upstream region was cloned into pCR-XL-TOPO	This study
pIH9	A ~1.3-kb KpnI/XhoI fragment from pIH8 was cloned into KpnI/SalI sites of pJIR751	This study
pIH10	An 883-bp DNA fragment containing the <i>C. difficile spo0A</i> ORF with an NdeI site inserted just before ATG was cloned into pCR-XL-TOPO	This study
pIH11	A 0.9-kb NdeI/PstI fragment from pIH10 was cloned into pIHOAPRO	This study
pIH12	A ~1.1-kb KpnI/XhoI fragment from pIH11 was cloned into KpnI/SalI sites of pJIR751	This study
pIH13	A 1,174-bp DNA fragment containing the <i>C. tetani spo0A</i> ORF and a ~400-bp upstream region was cloned into pCR-XL-TOPO	This study
pIH14	A ~1.2-kb KpnI/XhoI fragment from pIH13 was cloned into KpnI/SalI sites of pJIR751	This study
pIH15	A 744-bp DNA fragment containing the <i>C. perfringens spo0A</i> promoter region and the C-terminal domain with an NdeI site was cloned into pCR-XL-TOPO	This study
pIH16	A 459-bp DNA fragment containing the <i>C. botulinum spo0A</i> C-terminal domain with an NdeI site was cloned into pCR-XL-TOPO	This study
pIH17	A 386-bp DNA fragment containing the <i>C. difficile spo0A</i> C-terminal domain with an NdeI site was cloned into pCR-XL-TOPO	This study
pIH18	A ~0.8-kb NdeI/XbaI fragment from pIH15 was cloned into SpeI/NdeI sites of pIH16	This study
pIH19	A ~1.2-kb KpnI/XbaI fragment from pIH18 was cloned into KpnI/XbaI sites of pJIR751	This study
pIH20	A ~0.8-kb NdeI/XbaI fragment from pIH15 was cloned into SpeI/NdeI sites of pIH17	This study
pIH21	A ~1.1-kb KpnI/XbaI fragment from pIH20 was cloned into KpnI/XbaI sites of pJIR751	This study

TABLE 3. Sporulation of complemented strains grown in DS medium

Strain	Concn (CFU/ml) ^a of:		Frequency ^d
	Viable cells ^b	Spores ^c	
SM101	3.4×10^7	3.2×10^7	0.94
IH101	5.1×10^7	4.0×10^1	7.8×10^{-7}
IH101(pMRS123)	3.3×10^7	2.6×10^7	0.79
IH101(pIH2)	4.2×10^7	3.6×10^6	0.09
IH101(pIH4)	2.4×10^7	1.0×10^1	4.1×10^{-7}
IH101(pIH9)	3.1×10^7	1.0×10^1	3.1×10^{-7}
IH101(pIH14)	5.6×10^7	5.5×10^7	0.98
IH101(pIH7)	2.6×10^7	1.0×10^1	3.8×10^{-7}
IH101(pIH12)	2.8×10^7	2.4×10^2	8.6×10^{-6}
IH101(pIH19)	2.6×10^7	3.5×10^2	1.3×10^{-5}
IH101(pIH21)	1.2×10^7	3.3×10^2	2.9×10^{-5}

^a Results shown are based on at least three independent determinations for each experimental parameter for each culture.

^b Total CFU per milliliter present in each culture before heat treatment.

^c Total CFU per milliliter present in each culture after heat treatment at 80°C for 20 min.

^d Calculated as the ratio of the number of spores to the number of viable cells.

sequencing confirmed that no PCR-generated mutation was introduced during PCR amplification of the inserts. The recombinant fusion plasmids, pIH7, pIH12, pIH19, and pIH21, were introduced into IH101 by electroporation. Our RT-PCR analyses detected *spo0A*-specific RT-PCR products from RNAs of all four transformants, indicating that both wild-type and chimeric *spo0A* of *C. botulinum* and *C. difficile* were expressed in the *C. perfringens spo0A* mutant when placed under the control of the *C. perfringens spo0A* promoter. However, when the spore-forming capabilities of these transformants were compared with that of their host strain, IH101, no refractile endospores were detected after 8 h of growth in DS medium and no significant increase in heat-resistant spore formation was observed (Table 3.)

To determine if the inability of both wild-type and chimeric *spo0A* from *C. botulinum* and *C. difficile* to restore spore formation in IH101 resulted from a lack of Spo0A production, Western blot analysis was performed as previously described (18). Briefly, *C. perfringens* strains were grown in DS medium and sonicated until >95% of cells were lysed, and then each culture lysate was analyzed for the presence of Spo0A by using

FIG. 1. Western blot analysis of Spo0A production by wild-type *C. perfringens* and complemented strains. *C. perfringens* strains were grown in DS medium and sonicated as described elsewhere (10, 11). An aliquot (20 μ l) of each sonicated culture lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting with anti-*B. subtilis* Spo0A antibodies. The blot was subjected to chemiluminescence detection to identify immunoreactive species. (A) Results are shown for control strains SM101 (wild type) and IH101 (*spo0A* knockout mutant) and for representative complemented strains IH101(pMRS123), IH101(pIH7), IH101(pIH19), IH101(pIH12), and IH101(pIH21). (B) Results are shown for control strains SM101 (wild type) and IH101(pMRS123) and for complemented strains IH101(pIH2) and IH101(pIH14). The arrow on the left of the blot indicates the migration of the Spo0A-specific immunoreactive band.

an antibody against *B. subtilis* Spo0A. As expected, a Spo0A-specific immunoreactive band was observed in lysates prepared from SM101 and IH101(pMRS123), while no Spo0A-specific band was detected in lysates prepared from *spo0A* mutant IH101 (Fig. 1A). A Spo0A-specific immunoreactive band was also observed in sporulating culture lysates prepared from IH101 carrying wild-type or chimeric *spo0A* from *C. botulinum* or *C. difficile*, albeit at an intensity lower than those for SM101 and IH101(pMRS123) (Fig. 1A). To ensure that the lower intensity of the Spo0A-specific band observed was not due to a different reactivity to the antibody against *Bacillus* Spo0A, Western blot analysis of the two complemented strains IH101(pIH2) and IH101(pIH14) was also performed. The Spo0A-specific immunoreactive bands detected for both strains were similar in intensity to those of SM101 and IH101(pMRS123) (Fig. 1B). These results suggested that the inability of the wild-type and chimeric Spo0A from *C. botulinum* and *C. difficile* to restore spore formation in the *C. perfringens* *spo0A* mutant may be a result of decreased production of Spo0A.

We then performed Northern blot analyses, as previously described (10, 12), to evaluate whether the differential production of Spo0A observed was due to a differential level of *spo0A* mRNA synthesis. The *spo0A* transcripts detected for total RNA of IH101 carrying wild-type or chimeric *C. botulinum* or *C. difficile* *spo0A* were similar in intensity to each other but lower in intensity than that of IH101(pMRS123) (Fig. 2) (data not shown). When the relative *spo0A* mRNA levels were determined by semiquantitative Northern blot analysis, at least a fivefold difference in *spo0A* transcription was observed between IH101(pMRS123) and the four complementing strains IH101(pIH7), IH101(pIH12), IH101(pIH19), and IH101(pIH21) (Fig. 2) (data not shown). These results suggest that the lower level of Spo0A in IH101 carrying wild-type or chimeric Spo0A from *C. botulinum* or *C. difficile* may be a result of a decreased level of *spo0A* transcription.

FIG. 2. Comparative expression of *spo0A* mRNAs from IH101(pIH19) and IH101(pMRS123). Various amounts of RNA, extracted from IH101(pMRS123) and IH101(pIH19) cultures grown in DS medium at 37°C for 6 h as previously described (7, 10), were separated by electrophoresis on a 1% agarose gel and transferred by Northern blotting. The blots were hybridized with an AlkPhos-labeled *spo0A* probe, and hybridized probe was then detected by CDPstar chemiluminescence (Amersham Bioscience). The relative levels of *spo0A* mRNA in IH101(pIH19) were determined from a calibration curve, which was made using various amounts (2.0 to 8.0 μ g) of RNA prepared from the high-Spo0A-producing strain IH101(pMRS123). The densitometric analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The *spo0A* mRNA-specific band is indicated by an arrow. The various amounts of RNA loaded on the gel are given at the bottom of the blot.

Since CPE is a sporulation-regulated toxin, it was of interest to compare the levels of CPE production in the complemented strains. CPE Western blot analyses (6, 11) detected a ~35-kDa CPE-specific immunoreactive band in Western blots of lysates prepared from sporulating cultures of complemented strains IH101(pMRS123), IH101(pIH2), and IH101(pIH14) (data not shown). However, no CPE-specific immunoreactivity was detected in lysates prepared from complemented strains carrying wild-type or chimeric *spo0A* from *C. botulinum* or *C. difficile* grown under sporulation conditions (data not shown). Quantitative Western blot analyses (6, 15) showed similar levels of CPE production by complemented strains IH101(pIH14) and IH101(pMRS123) (data not shown). Although IH101(pIH2) produced a slightly smaller amount of CPE than IH101(pIH14) and IH101(pMRS123), this was not due to the influence of either total growth or sporulation (data not shown). Collectively, these results indicated that the lack of CPE production in the *C. perfringens* *spo0A* knockout mutant was restored by complementing the mutant with recombinant plasmids carrying wild-type *spo0A* from *C. acetobutylicum* or *C. tetani* but not from *C. difficile* or *C. botulinum*.

In summary, our results demonstrated that wild-type *spo0A* from *C. acetobutylicum* or *C. tetani*, but not from *C. botulinum* or *C. difficile*, restored sporulation and CPE production in a *spo0A* mutant of *C. perfringens*. The inability of wild-type and chimeric *spo0A* from *C. botulinum* or *C. difficile* to complement the sporulation defect of the *C. perfringens* *spo0A* mutant cannot be explained by the low level of amino acid sequence identity between Spo0A from *C. perfringens* and Spo0A from *C. botulinum* or *C. difficile*. Note that high levels of sequence identity were obtained when the amino acid sequence of Spo0A from *C. perfringens* was compared with those from *C. acetobutylicum* (76%), *C. botulinum* (78%), *C. difficile* (60%), and *C. tetani* (76%) (data not shown). The putative phosphorylation site (LDIIMP), the conformational switch region (KPFDP), and the helix-turn-helix (HTH) DNA-binding motif

FIG. 3. Comparison of amino acid sequences of phosphoacceptor and effector domains of Spo0A from *C. perfringens* (C.p), *C. acetobutylicum* (C.a), *C. botulinum* (C.b), *C. difficile* (C.d), and *C. tetani* (C.t). Asterisks indicate residues that are identical in all five species. Highlighting indicates possible active sites: SP, putative site of phosphorylation; CS, the conformational region; HTH, the helix-turn-helix motif. The deduced amino acid sequences of Spo0A from *C. perfringens*, *C. acetobutylicum*, and *C. tetani* were available on the Entrez Genome website (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome>). The amino acid sequences of *C. botulinum* and *C. difficile* were obtained from the Sanger Institute's website (<http://www.sanger.ac.uk/>). Amino acid alignment was performed using the ClustalW program and modified with Microsoft PowerPoint.

are also highly conserved in all *Clostridium* species compared (Fig. 3).

Our current results suggest that the lack of spore formation in IH101 complemented with *spo0A* from *C. botulinum* or *C. difficile* was due, at least in part, to the lower levels of *spo0A* transcription and Spo0A production, which were insufficient to initiate sporulation. However, it is not clear why lower levels of the *spo0A* transcript were detected with constructs where the *C. perfringens* *spo0A* promoter region was fused to *C. botulinum* or *C. difficile* *spo0A*. One possible explanation might be the instability of the *spo0A* transcript. Our results cannot exclude the possibility that certain unidentified amino acid differences within the DNA-binding domain of Spo0A from *C. botulinum* and *C. difficile* versus *C. perfringens* might be crucial for initiation of sporulation. Since a recent study (12) identified inorganic phosphate as an environmental signal for initiation of sporulation in *C. perfringens*, it is also possible that sporulation signals might be unique to each species and might reflect the environment where they reside (18).

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