

# Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A

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## Abstract

This study identified a functional *spo0A* ORF in enterotoxigenic *Clostridium perfringens* type A. To evaluate the function of *spo0A*, an isogenic *spo0A* knock-out mutant was constructed. The *spo0A* mutant was unable to form endospores and produce enterotoxin, however, these defects could be restored by complementing the mutant with a recombinant plasmid carrying the wild-type *spo0A* gene. These results provide evidence that *spo0A* expression is essential for sporulation and enterotoxin production in *C. perfringens*.

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**Keywords:** *C. perfringens*; Enterotoxin; *spo0A*; Sporulation

## 1. Introduction

Enterotoxigenic *Clostridium perfringens* type A are human gastrointestinal (GI) pathogens, causing food poisoning and non-food-borne human GI diseases [1]. Recent studies [2–4] have shown that *C. perfringens* type A food poisoning isolates carry the enterotoxin gene (*cpe*) on the chromosome, while *cpe* is located on a plasmid in non-food-borne GI disease isolates. Substantial experimental and epidemiological evidence [1,5] now indicates that most, if not all, GI symptoms of these *C. perfringens* associated diseases are caused by the *C. perfringens* enterotoxin (CPE). Although several studies [6–10] indicated that CPE synthesis and release is associated with sporulation, this association has never been confirmed by gene knock-out studies. Furthermore, the

molecular mechanism of sporulation and its role in CPE synthesis and release has not been studied in great detail at the molecular level. In this regard, we planned to investigate Spo0A, which belongs to a large family of bacterial proteins called response regulators [11]. The initiation of sporulation in *Bacillus subtilis* is controlled primarily by the phosphorylation state of the Spo0A protein [12]. The counterparts of *B. subtilis* Spo0A have been detected in many other *Bacillus* and *Clostridium* species [13,14], and recent studies [15,16] present evidence that *C. acetobutylicum* Spo0A transcriptionally activates the genes for sporulation and solvent formation. A *spo0A* homolog with a premature termination codon in the ORF was identified in the genome of the naturally *cpe*-negative *C. perfringens* strain 13 [17]. Our study reports the comparison of the *spo0A* ORF of *cpe*-positive isolates with that of strain 13, the construction of a *C. perfringens spo0A* knock-out mutant, and the effects of *spo0A* inactivation on sporulation and CPE production.

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## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

### 2.2. Cloning and sequencing of the *spo0A*-containing fragment from *cpe*-positive *C. perfringens* type A isolates

The 1037-bp DNA fragment from each of two chromosomal (NCTC8239 and SM101) and two plasmid (F4969 and B11) *cpe* isolates was PCR amplified using primers CPP29A (5'-GAGTGGATGTTAAAAG-ATGCA-3') and CPP29B (5'-GTGCTTTCTCCTTA-ATTTAGC-3'). These PCR products were then cloned into the pCR<sup>®</sup>-XL-TOPO<sup>®</sup> vector using the TOPO<sup>®</sup> XL cloning kit (Invitrogen). Both strands of the *spo0A*-containing DNA insert, from two clones for each isolate, were then sequenced using M13 forward and reverse primers.

### 2.3. Isolation of a *spo0A* knock-out mutant

The *spo0A* mutator plasmid was constructed as follows: An ~2.9-kb DNA fragment, carrying the *spo0A* ORF and ~1.0-kb each upstream and downstream region, was PCR amplified from SM101 using CPP5 (5'-GCAAGGGATTATTGTGAGAGT-3') and CPP6 (5'-CCTGGTGATGGTGCTATTTGT-3') and then cloned into pCR<sup>®</sup>-XL-TOPO<sup>®</sup> (Invitrogen) to create the plasmid pMRS110. Plasmid pMRS120 was then constructed by recloning the ~2.3-kb *Bgl*II–*Eco*RI frag-

ment of pMRS110 into the *Bam*HI–*Eco*RI sites of pMRS104 (Table 1). The *catP* gene was then inserted into the unique *Spe*I site located within the *spo0A* ORF in pMRS120 by: (i) digesting pMRS120 with *Spe*I; (ii) filling-in the resultant *Spe*I ends by a Klenow reaction, and (iii) blunt-end ligating a *Sma*I–*Nae*I fragment containing the *catP* gene from pJIR418 [18], to create pMRS121. This new plasmid contains an inactive *spo0A* gene and, as it contains no origin of replication for *C. perfringens*, is suicidal in this host. The *spo0A* mutator plasmid pMRS121 was used to transform, by electroporation [8], *C. perfringens* isolate SM101 to Em (50 µg/ml) and Cm (20 µg/ml) resistance and *spo0A* mutant was selected by allelic exchange using the protocol as previously described [5].

### 2.4. Preparation of digoxigenin (DIG)-labeled probes

An ~800-bp DIG-labeled *spo0A*-specific DNA probe was prepared by a previously described two-step PCR amplification method [5,8], using the primer set CPP29 (5'-AGCATGAAGGAATCAAAAATATCTG-3') and CPP29B (5'-GTGCTTTCTCCTTAATTTAGC-3'). The *catP* probe was produced using a 517-bp *Eco*RV–*Hpa*I fragment, containing internal *catP* gene sequences, from pJIR418. The vector probe was produced using an ~2.4-kb *Sma*I-fragment of pMRS104. These *catP*-and vector-containing DNA fragments were labeled using a Random Primed DNA Labeling system (Roche).

### 2.5. Southern blot analysis

Total DNA from wild type and *spo0A* mutant strains was isolated as previously described [5,8]. The

Table 1  
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Sources or Refs.
<i>C. perfringens</i>		
SM101	Electroporatable derivative of a food poisoning type A isolate NCTC8798, carries a chromosomal <i>cpe</i> gene	[10]
NCTC8239	Food poisoning type A isolate carries a chromosomal <i>cpe</i> gene	[2]
F4969	Non-food-borne GI disease isolate carries a plasmid borne <i>cpe</i> gene	[2]
B11	Non-food-borne GI disease isolate carries a plasmid borne <i>cpe</i> gene	[2]
IH101	<i>spo0A</i> knock-out mutant derivative of SM101	This study
<i>Plasmids</i>		
pJIR751	<i>C. perfringens</i> / <i>E. coli</i> shuttle vector; Em <sup>r</sup>	[20]
pJIR418	<i>C. perfringens</i> / <i>E. coli</i> shuttle vector; Cm <sup>r</sup> , Em <sup>r</sup>	[18]
pMRS103	<i>cpe</i> mutator plasmid, which contains <i>cpe::catP</i> allele	[5]
pMRS104	Constructed by digestion of pMRS103 with <i>Xba</i> I (which released <i>cpe::catP</i> ) and religation	This study
pMRS110	An ~2.9-kb PCR fragment, which contains the <i>spo0A</i> ORF and ~1.0-kb each upstream and downstream region, was cloned into pCR <sup>®</sup> -XL-TOPO <sup>®</sup>	This study
pMRS120	An ~2.3-kb <i>Bgl</i> II– <i>Eco</i> RI fragment from pMRS110 was cloned into pMRS104	This study
pMRS121	An ~1.3-kb <i>Sma</i> I– <i>Nae</i> I fragment of pJIR418, which contains chloramphenicol resistance determinant ( <i>catP</i> ), was incorporated into the unique <i>Spe</i> I site located in <i>spo0A</i> ORF in pMRS120	This study
pMW100	An ~1.0-kb PCR fragment, which contains <i>spo0A</i> ORF and ~200-bp upstream sequence, was inserted into pCR <sup>®</sup> -XL-TOPO <sup>®</sup>	This study
pMRS123	An ~1.0-kb <i>Kpn</i> I– <i>Xho</i> I fragment of pMW100 was cloned into <i>Kpn</i> I/ <i>Sal</i> I sites of pJIR751	This study

DNA was then digested with *HpaI* and three identical Southern blots were prepared using this digested DNA and hybridized, separately, with probes specific for the *spo0A*, *catP*, or pMRS104 sequences. The hybridized probe was detected using a DIG-chemiluminescence detection system, with CSPD substrate (Roche) as previously described [5].

### 2.6. RT-PCR analysis

*Clostridium perfringens* wild-type SM101, *spo0A* mutant IH101 and the complemented IH101(pMRS123) strains were grown in Duncan–Strong (DS) medium [7] at 37 °C for 6 h. These cultures were used to isolate total RNA as previously described [8,10]. The primers CPP68 (5'-CAGGAATTGCAAAGGATGGATTGGAAGC-3') and CPP69 (5'-GGCATCTATTTGTCCTCTTCCCCAAG-3'), which amplified a 619-bp internal *spo0A* fragment, were used to detect *spo0A*-specific mRNA in total RNA preparations by RT-PCR analysis with the commercially available Access RT-PCR kit (Promega).

### 2.7. Sporulation assay

*Clostridium perfringens* isolates were grown in fluid thioglycollate (FTG) medium [8] at 37 °C overnight. A 0.2 ml aliquot of the FTG grown culture was inoculated into 10 ml of DS medium [7] and allowed to grow at 37 °C for 8–24 h. These DS cultures were used to visualize refractile endospores using a phase-contrast microscope (Zeiss) as described previously [5,19]. The heat-resistant colony forming unit (CFU) present per ml of DS culture were determined by heating the culture at 80 °C for 20 min, plating the serially diluted samples on BHI agar plates and incubating anaerobically at 37 °C for 24 h.

### 2.8. CPE Western blot analysis

*Clostridium perfringens* strains grown in DS or FTG medium were sonicated until >95% of all cells were lysed (lysis was continuously monitored by phase-contrast microscopy). After sonication, each culture lysate was analyzed for the presence of CPE by Western blot analysis using a CPE antibody as previously described [5,7].

### 2.9. Nucleotide sequence accession numbers

The DNA sequences reported in this study have been deposited to GenBank and assigned Accession Nos: AY335913 (SM101), AY335914 (F4969), AY335915 (NCTC8239), and AY335916 (B11).

## 3. Results

### 3.1. Nucleotide sequencing analysis of *spo0A* in cpe-positive *C. perfringens* type A

Since *C. perfringens* strain 13 genome sequencing [17] identified a *spo0A* homolog with a premature termination codon in the ORF, we first evaluated whether cpe-positive *C. perfringens* type A isolates carry an intact *spo0A*. Nucleotide sequencing analyses (Fig. 1) revealed that no frame-shift mutations or premature termination codons were found in the *spo0A* ORF sequence of the two chromosomal (NCTC8239 and SM101) and two plasmid-borne (F4969 and B11) *cpe* isolates which we surveyed. However, the nucleotide substitution at position 452 (C instead of A) in all of our surveyed cpe-positive isolates replaces the termination codon TAA found in strain 13 by TCA, forming an intact ORF which encodes a protein of 276-aa homologous to Spo0A (Fig. 1). As in *B. subtilis* [14] and *C. acetobutylicum* [15], the *C. perfringens spo0A* putative promoter regions contain sequences that match promoter consensus sequences recognized by  $E\sigma^H$ . Contrary to *B. subtilis* [14] but similar to *C. acetobutylicum* [15], there is only one OA box, which overlaps the –10 elements of the putative  $E\sigma^H$  promoter, in the promoter regions of *C. perfringens spo0A* (Fig. 1).

### 3.2. Construction of *spo0A* knock-out mutant

The mutator plasmid pMRS121 was introduced into *C. perfringens* strain SM101 by electroporation, and transformants were selected on BHI agar plates containing Em and Cm. After several attempts, an Em<sup>r</sup> and Cm<sup>r</sup> transformant was obtained. PCR assay, using primers CPP68 and CPP69, yielded two products, of 619-bp and ~1.9-kb, from DNA of this transformant (data not shown). This result is consistent with the mutated *spo0A::catP* allele, present in pMRS121, being integrated into the chromosomal *spo0A* gene by a single cross-over event of homologous recombination (data not shown). This transformant was grown in non-selective conditions and a double crossover event between the wild-type *spo0A* and the mutated *spo0A::catP* allele was obtained after screening of ~3000 colonies for Cm<sup>r</sup>, and Em<sup>s</sup> phenotypes. The putative mutant was designated as IH101.

### 3.3. Molecular analysis of the *spo0A* knock-out mutant

Inactivation of *spo0A* in IH101 was first demonstrated by PCR analysis of DNA isolated from the mutant (Fig. 2(a)). Using *spo0A*-specific primers CPP68 and CPP69, a 619-bp *spo0A* internal fragment was amplified using template DNA isolated from wild-type

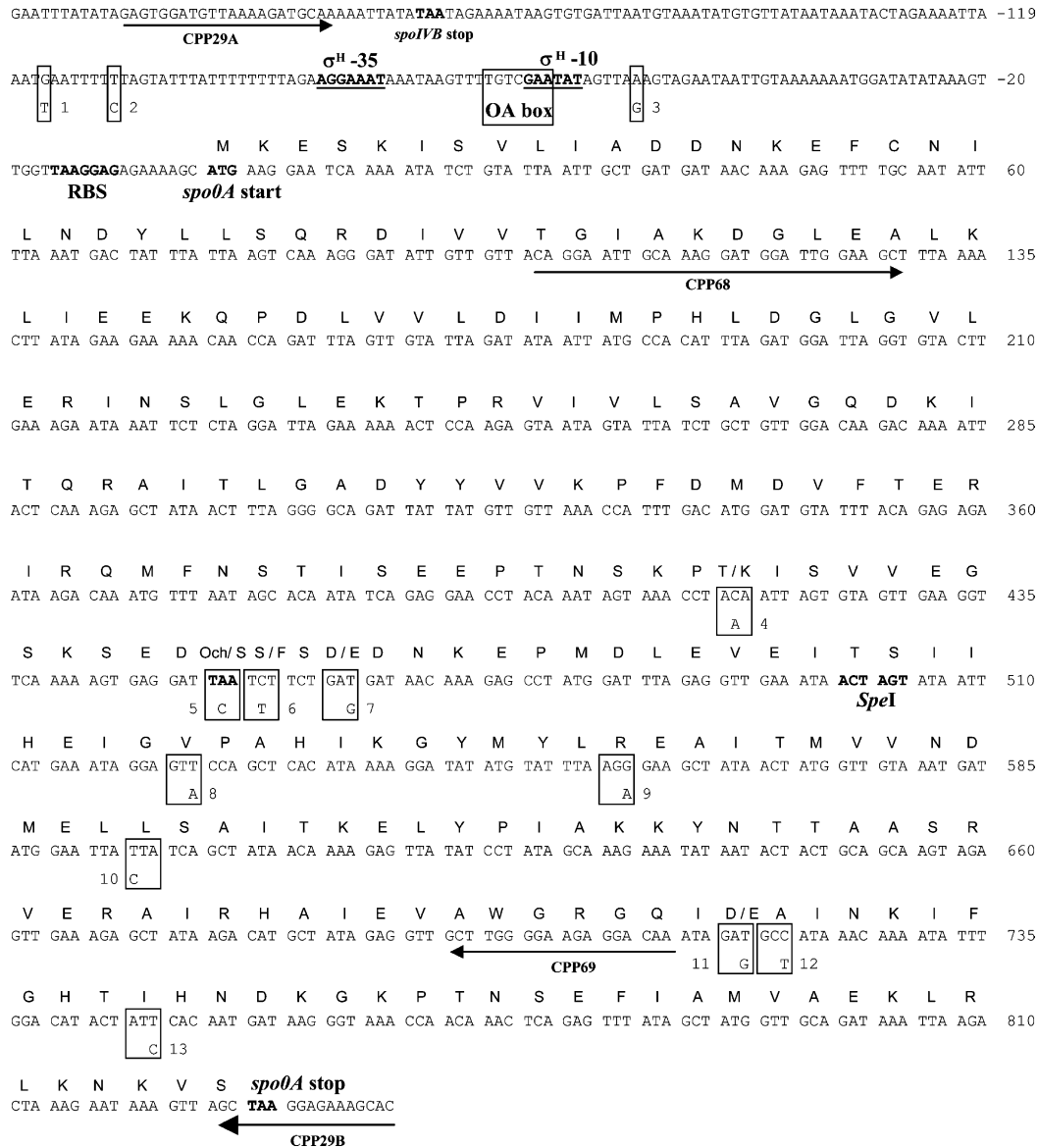


Fig. 1. Comparison between the *spo0A* sequence of *cpe*-positive *C. perfringens* type A strains and the *spo0A* sequence of strain 13. The consensus *spo0A* sequence present in *cpe*-positive isolates (lower line) was compared with the *spo0A* sequence of strain 13 (upper line). Only nucleotides of *cpe*-positive isolates that differ from the sequence of strain 13 are shown. Single letters on top of the nucleotide sequences indicate deduced amino acid sequences. RBS indicates the putative ribosome binding site,  $\sigma^H$ -10 and  $\sigma^H$ -35 indicate the -10 and -35 putative promoter consensus sequences recognized by  $E\sigma^H$ , OA box indicates sequence exhibiting a perfect match to OA box consensus (5'-TGTCGAA-3'), *spoIVB* stop indicates the location of *spoIVB* stop codon, CPP29A and CPP29B indicate the location of forward and reverse primers, respectively, used to amplify the 1037-bp PCR product for sequencing, CPP68 and CPP69 indicate the location of forward and reverse primers, respectively, used for RT-PCR analyses. The nucleotide substitutions in the sequences of *cpe*-positive isolates are shown by numbered boxes. Nucleotide substitutions in box 1 and box 3 are found in the *spo0A* upstream sequence of SM101 and the substitution in box 2 is found in *spo0A* upstream sequence of F4969. The substitution in box 5 (position 452) is found in the *spo0A* ORF of all four *cpe*-positive isolates. The substitution in box 9 is found in SM101 and B11. The substitution in box 10 is found in F4969 and B11. The substitution in box 12 is found in SM101 and F4969. The substitutions in boxes 4, 6, 7, 8, 11 and 13 are found only in the *spo0A* ORF sequence of SM101. Note that nucleotide substitutions in box 8, 9, 10, 12 and 13 are silent, i.e., these single base substitutions do not lead to an amino acid substitution.

strain SM101. In contrast, an ~1.9 kb PCR product was obtained using DNA isolated from mutant IH101 (Fig. 2(a)). These PCR results are consistent with the wild-type *spo0A* gene having been replaced with the mutated allele, which carries an extra ~1.3-kb *catP*-

containing fragment, present in mutator plasmid pMRS121. This conclusion receives further support from the observed amplification of a similar ~1.9 kb PCR product using pMRS121 as template DNA (data not shown).

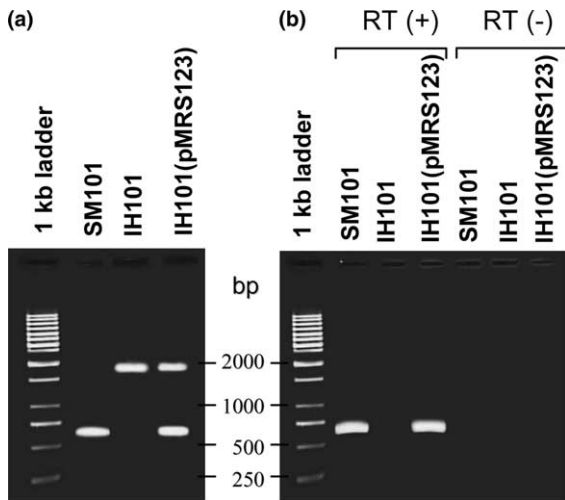


Fig. 2. PCR and RT-PCR analysis of *C. perfringens* strains. (a) Total DNA isolated from wild-type SM101, *spo0A* mutant IH101 and complemented IH101(pMRS123) strains was subjected to PCR analysis using *spo0A*-specific internal primers CPP68 and CPP69. The PCR amplified products were analyzed by agarose (1%) gel electrophoresis and photographed under UV light. Molecular sizes of the DNA markers (in bp) are given on the right. (b) Total RNA prepared from wild-type SM101, *spo0A* mutant IH101 and complemented IH101(pMRS123) strains were subjected to RT-PCR analysis using *spo0A*-specific internal primers CPP68 and CPP69. RT (+) and RT (-) indicate the presence or absence, respectively, of reverse transcriptase in the RT-PCR. The RT-PCR amplified products were analyzed by agarose (1%) gel electrophoresis and photographed under UV light. Molecular sizes of the DNA markers (in bp) are given on the left.

Southern blot analyses (data not shown) showed that an ~8.5-kb *HpaI* DNA fragment from wild-type strain SM101 hybridized with our *spo0A*-specific probe. However, two hybridizing bands, of ~6 and ~4 kb, were observed with DNA from mutant strain, IH101. This profile is consistent with results expected since the ~1.3-kb *catP*-containing fragment has an internal *HpaI* site. Our *catP*-specific probe hybridized with an ~6-kb *HpaI* fragment of IH101 DNA, but as expected, no hybridizing band was observed with DNA from wild-type SM101. A final piece of evidence supporting the predicted double crossover event between the wild-type *spo0A* and the *spo0A::catP* segment from pMRS121 is the observation that the vector pMRS104-specific probe did not hybridize with IH101 DNA.

#### 3.4. Evaluation of *spo0A* expression by *spo0A* knock-out mutant

Next, we confirmed by RT-PCR analyses that the *spo0A* knock-out mutant IH101 is unable to express *spo0A*. As expected, a 619-bp amplified product was detected in RNA of wild-type strain SM101 in the presence of RT (Fig. 2(b)). The size of the RT-PCR amplified product exactly matched the size of product

obtained in the control PCR reaction with SM101 DNA using the same primers (Fig. 2(a)). These results indicated that the *spo0A* ORF present in wild-type SM101 is transcriptionally active. When the same RT-PCR analyses were applied to RNA of the *spo0A* mutant IH101, no 619-bp RT-dependent product was detected (Fig. 2(b)), indicating that IH101 is unable to produce an intact *spo0A*-specific transcript. The absence of the signal is consistent with a scenario where the mutated locus is transcribed but the transcript is terminated upstream of the binding site for primer CPP69, i.e., within the insertion at the *SpeI* site (Fig. 1).

#### 3.5. Effect of *spo0A* inactivation on sporulation of *C. perfringens*

Like *spo0A* mutants of *B. subtilis*, *B. anthracis* and *C. acetobutylicum* [14], isolated colonies formed by *C. perfringens spo0A* knock-out mutant IH101 were flatter, more translucent and had more irregular edges than those formed by wild-type strain SM101, consistent with a pleiotropic early sporulation block (data not shown). However, no  $Spo^-$  colony morphology was observed with the mutant complemented with a recombinant plasmid pMRS123 (Table 1) carrying the wild-type *spo0A* gene. These results indicated that the  $Spo^-$  colony morphology of the *spo0A* knock-out mutant was due to the specific inactivation of the *spo0A* gene.

When the sporulation capability of the mutant was compared with that of its wild-type parent in liquid DS medium, the wild-type strain SM101 exhibited significant sporulation, i.e., refractile endospores were visualized by phase-contrast microscopy (Fig. 3) after 8 h of growth. However, the *spo0A* knock-out mutant IH101 remained asporogenous (Fig. 3), i.e., no refractile endospores were observed in DS culture of IH101 even after 24 h of growth. When similar phase-contrast microscopic examination was performed on the complemented strain [IH101(pMRS123)], a wild-type level of sporulation was observed in DS culture (Fig. 3).

To further confirm the  $Spo^-$  phenotype of the *spo0A* knock-out mutant, the heat-resistant spore forming capability of the *spo0A* mutant was compared with that of the wild-type strain. The *spo0A* mutant IH101 exhibited significantly decreased production of heat-resistant spores compared to wild-type SM101 (Table 2). This lack of ability of the *spo0A* knock-out mutant to form heat-resistant spores could be complemented by the recombinant plasmid pMRS123 carrying the wild-type *spo0A* gene (Table 2).

Collectively, these results confirmed that the loss of a sporulation phenotype by the *spo0A* knock-out mutant

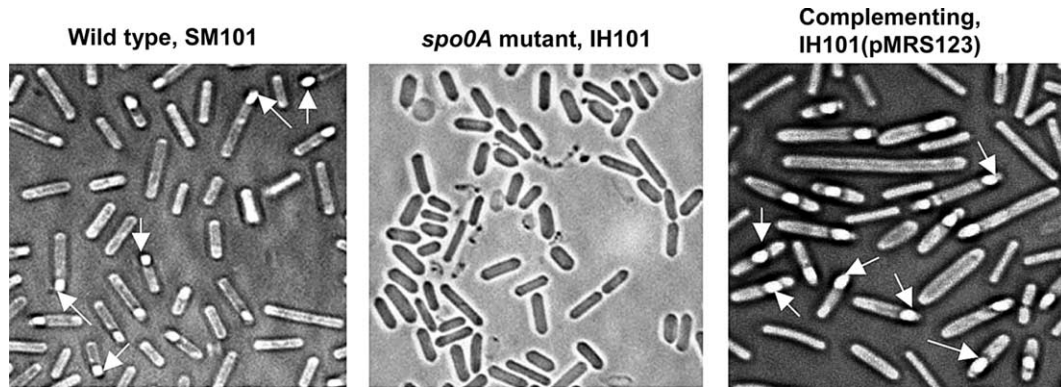


Fig. 3. Phase-contrast microscopic analysis of sporulating *C. perfringens* cultures. *C. perfringens* wild-type SM101, *spo0A* knock-out mutant IH101 and complemented IH101(pMRS123) strains were grown in DS medium at 37 °C for 8–24 h and refractile endospores were visualized using a phase-contrast microscope (Zeiss) with 1000× magnification. Representative fields were photographed at 1000× magnification. Note that refractile endospores (indicated by arrows) were observed in 8h-grown DS cultures of both SM101 and IH101(pMRS123), however, no detectable spores were found in DS culture of *spo0A* knock-out mutant IH101 even after 24 h of growth.

Table 2  
Sporulation of *C. perfringens* strains grown in DS medium

Strain	CFU/ml <sup>a</sup>		Frequency <sup>d</sup>
	Viable cells <sup>b</sup>	Spores <sup>c</sup>	
SM101	$3.0 \times 10^7$	$2.8 \times 10^7$	0.93
IH101	$5.6 \times 10^7$	$7.0 \times 10^1$	$1.2 \times 10^{-6}$
IH101(pMRS123)	$4.3 \times 10^7$	$3.5 \times 10$	0.81

<sup>a</sup> Results shown are based upon at least three independent determinations for each experimental parameters for each culture.

<sup>b</sup> Viable cells represents total CFU/ml present in each culture before heat treatment.

<sup>c</sup> Spores represents total CFU/ml present in each culture after heat treatment at 80 °C for 20 min.

<sup>d</sup> Frequency is calculated as the ratio of the number of spores to the number of viable cells.

was caused by the specific inactivation of the *spo0A* gene and the resultant loss of Spo0A production.

### 3.6. Effect of *spo0A* inactivation on *cpe* production

In order to determine whether or not Spo0A production, and hence endospore formation, is essential for CPE production, we compared the CPE producing capabilities of the wild-type strain SM101 and *spo0A* knock-out mutant IH101. An ~35-kDa CPE-specific immunoreactive band was detected in Western blots of lysates prepared from sporulating cultures of SM101 (Fig. 4), whereas no CPE-specific immunoreactivity was detected in lysates prepared from IH101 cultures grown in sporulating conditions. However, an ~35-kDa immunoreactive band, which co-migrated with the CPE-specific band of wild-type strain SM101, was observed in sporulating culture lysates of complemented strain IH101(pMRS123) (Fig. 4). These results indicated that the lack of CPE production in the *spo0A* knock-out mutant was due to the specific inactivation of the *spo0A* gene.

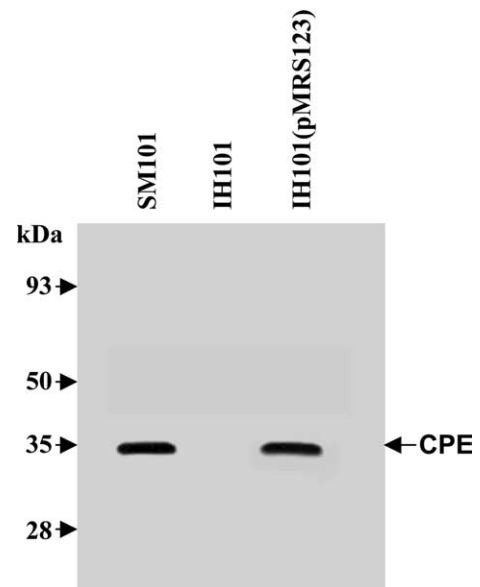


Fig. 4. Western blot analysis of CPE production by wild-type, mutant and complemented strains. *C. perfringens* wild-type SM101, *spo0A* mutant IH101 and complemented IH101(pMRS123) strains were grown in DS medium and sonicated as described in Section 2. An aliquot (25 µl) of each sonicated culture lysate was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting with CPE antibodies. The blot was subjected to chemiluminescence detection to identify immunoreactive species. Molecular mass markers (in kDa) are shown on the left; the arrow on the right indicates the migration of CPE-specific immunoreactive bands.

## 4. Discussion

This study demonstrated the presence of a functional *spo0A* gene in *cpe*-positive *C. perfringens* type A isolates. Nucleotide sequencing revealed that the *spo0A* ORFs in our surveyed *cpe*-positive isolates are intact and encode a protein of 276 aa with high conservation in the regions showing functional features of Spo0A from other bac-

teria [13–15] suggesting that the *C. perfringens* Spo0A is functional. This received support from our RT-PCR analyses which demonstrated that SM101 *spo0A* was expressed during sporulation. Although we could not demonstrate the production of Spo0A by Western blotting due to unavailability of Spo0A-specific antibody, our RT-PCR analyses showed that the RT-dependent transcript obtained with RNA of wild-type SM101 is specific for the expression of *spo0A* because our *spo0A* mutant IH101 was unable to produce a *spo0A*-specific transcript, and this lack of transcription could be complemented by a recombinant plasmid carrying the wild-type *spo0A* gene.

In the current study, the inactivation of the *spo0A* gene dramatically affected spore formation and CPE production capability of SM101 in laboratory sporulation conditions. Our results indicate that *spo0A* expression is essential for the production of refractile, heat-resistant spores by SM101. This claim is supported by the failure of DS culture of IH101 to produce any visible refractile spores and the restoration of this defect in the complemented strain IH101(pMRS123). Further support came from our observation that IH101 failed to produce a significant number of heat-resistant spores, while spores were obtained with complemented strain IH101(pMRS123) at a frequency similar to the wild-type. Given the association between sporulation and CPE production, our findings that CPE production was absent in DS culture lysates of our asporogenous *spo0A* knock-out mutant and reversion of this effect by complementing the mutant with a recombinant plasmid carrying the wild type *spo0A* gene, provide direct genetic evidence supporting the strong linkage between sporulation and CPE production. The mechanism of Spo0A-regulated CPE synthesis remains unknown. However, two hypotheses can be envisioned: (i) Spo0A may activate transcription of the *cpe* gene via activating sporulation-specific sigma factors encoding genes, *sigE* and *sigK*, and/or (ii) Spo0A directly activates *cpe* by binding to the putative 0A box (TGTAGAA) located in the promoter region of the *cpe* gene [9,10]. Further studies of Spo0A and *cpe* promoter binding, and *sigE* and *sigK* knock-out mutants, should help in understanding the mechanism of Spo0A-regulated CPE synthesis.

To our knowledge, this report represents the first successful study involving the construction of a *C. perfringens* sporulation gene knock-out mutant. The greatest challenge faced in our study was the lack of an easy screening method for the second cross-over event. To overcome this screening problem, we used our previously described [5] double-antibiotic selection strategy. Our present study also validates that this approach, which involves screening double cross-over events by monitoring for a Cm<sup>r</sup>, and Em<sup>s</sup>, phenotype should have widespread applicability for constructing other gene knock-outs in *C. perfringens*. Finally, this report pro-

vides an invaluable tool, the *spo0A* knock-out mutant, to probe sporulation processes in *C. perfringens* at the molecular level using SM101 DNA microarray.

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