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.
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'-GAGTGGGATTAAAGATGCA-3') and CPP29B (5'-GTGCTTTCCTATGATGCA-3'). These PCR products were then cloned into the pCR®-XL-TOPO® vector using the TOPO® 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 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'-AGCATGAAGGAATCATGCA-3') and CPP29B (5'-GTGCTTTCCTATGAGC-3'). The catP probe was produced using a 517-bp EcoRV–HpaI fragment, containing internal catP gene sequences, from pJIR418. The vector probe was produced using an ~2.4-kb SmalI 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

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Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Sources or Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. perfringens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM101</td>
<td>Electroporatable derivative of a food poisoning type A isolate NCTC8798, carries a chromosomal cpe gene</td>
<td>[10]</td>
</tr>
<tr>
<td>NCTC8239</td>
<td>Food poisoning type A isolate carries a chromosomal cpe gene</td>
<td>[2]</td>
</tr>
<tr>
<td>F4969</td>
<td>Non-food-borne GI disease isolate carries a plasmid borne cpe gene</td>
<td>[2]</td>
</tr>
<tr>
<td>B11</td>
<td>Non-food-borne GI disease isolate carries a plasmid borne cpe gene</td>
<td>[2]</td>
</tr>
<tr>
<td>IH101</td>
<td>spo0A knock-out mutant derivative of SM101</td>
<td>This study</td>
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<td></td>
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<td></td>
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<tr>
<td>Plasmids</td>
<td></td>
<td></td>
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<td>pJIR751</td>
<td>C. perfringens/E. coli shuttle vector; Em'</td>
<td>[20]</td>
</tr>
<tr>
<td>pJIR418</td>
<td>C. perfringens/E. coli shuttle vector; Cm', Em'</td>
<td>[18]</td>
</tr>
<tr>
<td>pMRS103</td>
<td>cpe mutator plasmid, which contains cpe:catP allele</td>
<td>[5]</td>
</tr>
<tr>
<td>pMRS104</td>
<td>Constructed by digestion of pMRS103 with XbaI (which released cpe:catP) and religation</td>
<td>This study</td>
</tr>
<tr>
<td>pMRS110</td>
<td>An ~2.9-kb PCR fragment, which contains the spo0A ORF and ~1.0-kb each upstream and downstream region, was cloned into pCR®-XL-TOPO®</td>
<td>This study</td>
</tr>
<tr>
<td>pMRS120</td>
<td>An ~2.3-kb BglII–EcoRI fragment from pMRS110 was cloned into pMRS104</td>
<td>This study</td>
</tr>
<tr>
<td>pMRS121</td>
<td>An ~1.3-kb SmalI–Nael fragment of pJIR418, which contains chloramphenicol resistance determinant (catP), was incorporated into the unique SpeI site located in spo0A ORF in pMRS120</td>
<td>This study</td>
</tr>
<tr>
<td>pMW100</td>
<td>An ~1.0-kb PCR fragment, which contains spo0A ORF and ~200-bp upstream sequence, was inserted into pCR®-XL-TOPO®</td>
<td>This study</td>
</tr>
<tr>
<td>pMRS123</td>
<td>An ~1.0-kb KpnI–XhoI fragment of pMW100 was cloned into KpnI/SalI sites of pJIR751</td>
<td>This study</td>
</tr>
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</table>
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 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'-CAGGAATTCAAGGATGGATTGGAAGC-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 cepo-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 cepo-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) cepo isolates which we surveyed. However, the nucleotide substitution at position 452 (C instead of A) in all of our surveyed cepo-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 EσII. Contrary to *B. subtilis* [14] but similar to *C. acetobutylicum* [15], there is only one 0A box, which overlaps the −10 elements of the putative EσII 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 Emr and Cms 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′, and Em′ 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...
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).
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 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
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.

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>CFU/mla</th>
<th>Frequencyd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viable cells</td>
<td>Spores</td>
</tr>
<tr>
<td>SM101</td>
<td>3.0 × 10⁷</td>
<td>2.8 × 10⁷</td>
</tr>
<tr>
<td>IH101</td>
<td>5.6 × 10⁷</td>
<td>7.0 × 10⁷</td>
</tr>
<tr>
<td>IH101(pMRS123)</td>
<td>4.3 × 10⁷</td>
<td>3.5 × 10⁷</td>
</tr>
</tbody>
</table>

*a* Results shown are based upon at least three independent determinations for each experimental parameters for each culture.

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

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

*d* Frequency is calculated as the ratio of the number of spores to the number of viable cells.

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-

![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.](https://academic.oup.com/femsle/article-abstract/233/2/233/576004)

![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.](https://academic.oup.com/femsle/article-abstract/233/2/233/576004)
teria [13–15] suggesting that the \textit{C. perfringens} Spo0A is functional. This received support from our RT-PCR analyses which demonstrated that SM101 \textit{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 \textit{spo0A} because our \textit{spo0A} mutant IH101 was unable to produce a \textit{spo0A}-specific transcript, and this lack of transcription could be complemented by a recombinant plasmid carrying the wild-type \textit{spo0A} gene.

In the current study, the inactivation of the \textit{spo0A} gene dramatically affected spore formation and CPE production capability of SM101 in laboratory sporulation conditions. Our results indicate that \textit{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 \textit{spo0A} knock-out mutant and reversion of this effect by complementing the mutant with a recombinant plasmid carrying the wild type \textit{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 \textit{cpe} gene via activating sporulation-specific sigma factors encoding genes, \textit{sigE} and \textit{sigK}, and/or (ii) Spo0A directly activates \textit{cpe} by binding to the putative 0A box (TGTAGAA) located in the promoter region of the \textit{cpe} gene [9,10]. Further studies of Spo0A and \textit{cpe} promoter binding, and \textit{sigE} and \textit{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 \textit{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 \textit{Cm}\textsuperscript{r} and \textit{Em}\textsuperscript{r} phenotype should have widespread applicability for constructing other gene knock-outs in \textit{C. perfringens}. Finally, this report provides an invaluable tool, the \textit{spo0A} knock-out mutant, to probe sporulation processes in \textit{C. perfringens} at the molecular level using SM101 DNA microarray.

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**References**


