RelA/DTD-mediated regulation of spore formation and toxin production by Clostridium perfringens type A strain SM101

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Abstract
RelA is a global regulator for stationary phase development in the model bacterium Bacillus subtilis. The relA gene forms a bicistronic operon with the downstream dtd gene. In this study, we evaluated the significance of RelA and DTD proteins in spore formation and toxin production by an important gastrointestinal pathogen Clostridium perfringens. Our β-glucuronidase assay showed that in C. perfringens strain SM101, relA forms a bicistronic operon with its downstream dtd gene, and the relA promoter is expressed during both vegetative and sporulation conditions. By constructing double relA dtd and single dtd mutants in C. perfringens SM101, we found that: (1) RelA is required for maintaining the efficient growth capacity of SM101 cells during vegetative conditions; (2) both RelA and DTD are required for spore formation and enterotoxin (CPE) production by SM101; (3) RelA/DTD activate CodY, which is known to activate spore formation and CPE production in SM101 by activating a key sporulation-specific σ factor F; (4) as expected, RelA/DTD activate sporulation-specific σ factors (σF, σE, σG and σ2) by positively regulating Spo0A production; and finally (5) RelA, but not DTD, negatively regulates phospholipase C (PLC) production by repressing plc gene expression. Collectively, our results demonstrate that RelA modulates cellular physiology such as growth, spore formation and toxin production by C. perfringens type A strain SM101, although DTD also plays a role in these pleiotropic functions in coordination with RelA during sporulation. These findings have implications for the understanding of the mechanisms involved in the infectious cycle of C. perfringens.

INTRODUCTION

Clostridium perfringens is a species of a Gram-positive, anaerobic, spore-forming bacteria that causes severe gastrointestinal (GI) and histotoxic infections in humans and animals. Although approximately 20 different C. perfringens toxins have been reported, C. perfringens strains are commonly classified into one of five types (A to E) based on their production of four major toxins: alpha-, beta-, epsilon- and iota-toxins [1, 2]. All strains produce alpha-toxin [CPA or phospholipase C (PLC)], which is recognized as a major virulence factor for gas gangrene and histotoxic infections in humans [3]. Some type A stains also produce C. perfringens enterotoxin (CPE), a major virulence factor for food poisoning (FP) and non-food-borne (NFB) GI diseases in humans [1]. In addition to toxin production, C. perfringens have the ability to form heat-resistant spores, which are the persistent and infectious forms of this bacterium and are responsible for the transmission of C. perfringens-associated diseases [4, 5]. When growth-promoting food sources are favourable, heat-resistant spores of C. perfringens FP isolates can germinate and outgrow into vegetative cells. After ingestion of contaminated foods, C. perfringens vegetative cells undergo sporulation followed by CPE production, leading to CPE-mediated cramping and diarrhea in the human gut [1, 6]. C. perfringens-associated gas gangrene and other NFB GI illnesses, such as antibiotic-associated diarrhea, occur when spores undergo germination upon contact with the host, followed by outgrowth, cell proliferation and toxin secretion [7].

The molecular regulation of spore formation by C. perfringens has not been studied as thoroughly as that of Bacillus subtilis, the best-characterized spore-forming bacteria. Previous studies demonstrated that initiation of spore formation and CPE production by C. perfringens strain SM101 is primarily controlled by Spo0A, a master regulator of sporulation [8]. Phosphorylated Spo0A triggers a cascade of sporulation-specific sigma (σ) factors, and σF controls the production of three
other σ factors (σE, σG and σK) required for C. perfringens spore formation and CPE production [9–11]. A study using C. perfringens type D strain CN3718 demonstrated that CodY negatively regulates spore formation in this strain by repressing spo0A transcription, as it does in Clostridium difficile and B. subtilis [12–14]. However, in contrast to these findings, a study using B. subtilis revealed that CodY actually activates spore formation by activating spo0A transcription [15]. Similarly, a recent study with C. perfringens also reported that CodY promotes spore formation and CPE production in C. perfringens type A FP strain SM101 by activating early sporulation-specific σF [16].

The activity of CodY is controlled by the transient increase in hyperphosphorylated guanosine nucleotides [(p)ppGpp] through a stringent response [17]. In B. subtilis, (p)ppGpp production is primarily determined by the net activity of RelA, a bifunctional (p)ppGpp synthetase/hydrolase; and two monofunctional (p)ppGpp synthetases, YwaC and YjbM [18]. The ppGpp-deficient (relA ywaC yjbM) strain had a delay in the rise of stationary phase induction of spo0A transcription. The relA mutant, which is expected to accumulate excess (p)ppGpp, has a more extreme effect, suggesting that excess (p)ppGpp is inhibitory and that this molecule may function optimally in sporulation within a narrow concentration range [15, 19]. The relA and triple relA yjbM ywaC mutants also had a delay in sporulation, and had 20- and 50-fold lower sporulation frequencies than the wild-type strain, respectively [15]. Furthermore, the quadruple relA yjbM ywaC codY mutant failed to form any spores, which contradicts the hypothesis that sporulation is initiated when a (p)ppGpp-induced depression of GTP relieves repression by CodY [14, 19].

In B. subtilis, relA forms a bicistronic operon with its downstream gene dtd, which encodes d-tyrosyl-tRNA Tyr deacylase (DTD), an enzyme that prevents the misincorporation of d-amino acids that are toxic to many organisms [20–22]. Although extensive research has been conducted on RelA in B. subtilis, few studies have been conducted on DTD, possibly because many B. subtilis laboratory strains, including B. subtilis 168, harbour a truncated DTD lacking 24 residues from the N-terminal domain [23].

Genome sequencing identified a RelA-DTD homologue in the genome of C. perfringens strains, including strain SM101 [24]. In this study, by constructing and characterizing relA and dtd mutants, we provide evidence that RelA modulates cellular physiology such as growth, sporulation and toxin production by C. perfringens type A strain SM101, although DTD also plays a role in these pleiotropic functions in coordination with RelA during sporulation.

**METHODS**

**Bacterial strains and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. We used the following growth media to propagate C. perfringens strains: fluid thioglycolate (FTG) broth (Becton Dickinson, NJ), TGY (3 % tryptase, 2 % glucose, 1 % yeast extract, 0.1 % l-cysteine) broth [25], Duncan–Strong (DS) sporulation medium [26], brain heart infusion (BHI) agar (Becton Dickinson), and egg yolk agar (HiMedia Laboratories, Mumbai, India) plates. Liquid cultures were incubated in a 37 °C water bath without shaking. Plate cultures were incubated in anaerobic jars containing GasPak (BD) at 37°C. *Escherichia coli* DH5α was cultured on Luria–Bertani (LB) agar plate or in LB broth (Becton Dickinson).

**Construction of gusA-fusion plasmids and β-glucuronidase assay**

The DNA fragment carrying the upstream region of each of relA, dtd and CPR_1902 from C. perfringens strain SM101 was amplified by PCR using primer pairs CPP1080/CPP1081, CPP1328/CPP1083 and CPP1084/CPP1085 (Table S1, available in the online version of this article), respectively. The SalI and PstI restriction sites were incorporated in the forward and reverse primers, respectively, of each primer pair. PCR products were then cloned into the pCR-XL-TOPO vector (Thermo Fisher Scientific). The recombinant clones carrying the expected DNA fragment were confirmed by restriction enzyme digestion, PCR analysis and DNA sequencing. The SalI-PstI fragments carrying 430, 546 or 416 bp upstream regions of relA, dtd or CPR_1902, respectively, from pCR-XL-TOPO clones were then re-cloned into the SalI-PstI site of pMRS127, an E. coli-C. perfringens shuttle vector [5], to create pCRrelA∗, pCRdtd∗ or pCR_CPR_1902_gusA fusion constructs, pPKT84, pPK8 or pPKT86, respectively (Fig. 1). These plasmids were introduced into C. perfringens strain SM101 by electroporation [27], and erythromycin-resistant (Em+) transformants were selected. The SM101 transformants carrying pCRrelA∗, pCRdtd∗ or pCR_CPR_1902_gusA fusions were grown in TGY (vegetative condition) and DS (sporulation condition) broth, and tested for β-glucuronidase (GUS) activity as previously described [5].

**Construction of relA and dtd mutants**

The relA and dtd genes of C. perfringens SM101 were inactivated using TargeTron Gene Knockout System (Sigma-Aldrich) as described previously [28] (Fig. 2a). The target sites in the relA and dtd genes were designed between nucleotides 687 and 688, and 381 and 382, respectively, from the start codon using the online design tool of The University of Nottingham, UK [29]. The pJR3566 [30] derivative plasmids, pRS2 and pRS6, in which TargeTron has been respectively retargeted to relA and dtd, were introduced into C. perfringens SM101 by electroporation, and Em+ transformants were then selected on BHI agar plates with 50 µg ml^-1 Em. The Em+ transformants were screened by colony PCR for an approximately 2 kb increase in the band size of relA using primers CPP1280 and CPP1281, and of dtd using primers CPP1268 and CPP1271 (Fig. 2b and Table S1). To cure pJR3566 harboured chloramphenicol resistance (Cm+), relA TargeTron and dtd TargetTron clones were sub-cultured three times in FTG broth and then plated onto BHI agar, and single colonies were patched onto BHI agar.
Table 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
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<th>Source or reference</th>
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<td>SM101 <em>relA::ermB</em></td>
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<td>SM101 <em>dtd::ermB</em></td>
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<td>416 bp upstream region of CPR, 1902 fused with <em>gusA</em> in pMRS127</td>
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<td>pRS2</td>
<td>–350 bp <em>BsrGI</em>-HindIII fragment retargeted to <em>relA</em> cloned between <em>BsrGI</em>-HindIII sites of pJIR3566</td>
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<td>pRS3</td>
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<td>pRS4</td>
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agar with Cm or Em, giving strains RS102 (*relA*) and RS103 (*dtd*), respectively.

**Construction of complemented strains**

To construct the *relA* complementation construct, a 2.6 kb DNA fragment carrying the 430 bp region upstream of *relA*, the *relA* ORF and the 60 bp region downstream was amplified from the DNA of strain SM101 using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) with primers CPP1106-KpnI and CPP1107-Sall (Table S1). To construct the *relA-dtd* operon complementation construct, a 3.2 kb DNA fragment carrying the 430 bp region upstream of *relA*, the *relA* ORF, the *dtd* ORF and the 197 bp region downstream was amplified using primers CPP1106-KpnI and CPP1108-Sall (Table S1). These 2.6 and 3.2 kb PCR fragments were digested with KpnI and Sall and then cloned between the KpnI and Sall restriction sites of *C. perfringens*-*E. coli* shuttle multicopy plasmid pJIR750 [31], yielding plasmids pRS3 and pRS4, respectively. Plasmids pRS3 and pRS4 were introduced into the *C. perfringens* *relA* mutant (strain RS102) by electroporation, and Em’ Cm’ transformants were selected, yielding strains RS102(pRS3) and RS102(pRS4), respectively.

To construct the plasmid carrying *dtd* fused with the *relA* promoter, primers CPP1080-Sall and CPP1081-PstI (Table S1) were used to amplify the 430 bp region upstream of *relA* and the 5’-fragment (81 bp) of the *relA* ORF using SM101 DNA. The resulting PCR fragment was digested with Sall and PstI, and cloned between the Sall and PstI restriction sites of pJIR750 [31], yielding plasmid pRS10. Primers CPP1330-PsrI and CPP1331-HindIII (Table S1) were used to amplify a DNA fragment carrying the 21 bp region upstream of *dtd*, the *dtd* ORF and the 197 bp region downstream of *dtd*. The resulting PCR fragment was digested with PstI and HindIII, and cloned between the PstI and HindIII restriction sites of pRS10, yielding plasmid pRS11, which generates a stop codon in the 5’-fragment (81 bp) of the *relA* ORF. pRS11 was introduced into *C. perfringens* *relA* (strain RS102) and *dtd* (strain RS103) mutants by electroporation, and Em’ Cm’ transformants were selected, yielding strains RS102(pRS11) and RS103(pRS11), respectively.
Growth kinetics
Then, 2 h TGY pre-cultured C. perfringens strains were inoculated into fresh 10 ml TGY (OD$\text{600}$=0.01 to 0.02). These cultures were incubated at 37°C, and the growth kinetics were determined by measuring the OD$\text{600}$ of the culture for up to 12 h. These procedures were repeated at least three times.

Sporulation efficiency
Overnight DS cultures of C. perfringens strains were heated at 80°C for 10 min to kill any vegetative cells. Appropriate volumes of the serially diluted heated or unheated cultures, which represent heat-resistant cells or total cells, respectively, were plated onto BHI agar, and colonies were counted after a 24 h incubation to quantify heat-resistant spores. Sporulation efficiency was determined based on the ratio of heat-resistant cells/total cells for each strain compared to the wild-type.

RNA extraction and quantitative RT-PCR (qRT-PCR)
Total RNA was extracted from C. perfringens strains grown for 5 h at 37°C in TGY or DS using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). RNA (3 µg) was treated with 6 U DNase I (Thermo Fisher Scientific) at 37°C for 1 h and the enzyme was inactivated by adding 3 µl of 50 mM EDTA followed by incubation at 65°C for 10 min. Samples were tested for genomic DNA contamination using PCR for 16S rRNA. A cDNA synthesis was performed with reverse transcription reaction mixture containing 200 ng RNA and random hexamers using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

qRT-PCR assays were performed in triplicate using iQ SYBR Green Supermix (Bio-Rad). Each reaction contained 5 µl master mix, 250 nM of each primer, 1 µl cDNA template (20-fold dilution) and nuclease-free water, to a final volume of 10 µl. All primers were designed using the Primer 3 website (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and listed in Table S1. Amplification and detection were performed using CFX96 Real-Time PCR Detection System (Bio-Rad) as follows: an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 57°C for 30 s, and a melting curve analysis form 60 to 95°C. Transcript levels were normalized to the housekeeping gene 16S rRNA and calculated by the comparative threshold cycle (Ct) ($2^{-\Delta\Delta Ct}$) method.

Western blot analysis
For Western blot analysis of SpoA, C. perfringens strains were grown in DS for 12 h at 37°C, and 400 µl of this culture
was inoculated and propagated in 20 ml DS for 5 h at the same temperature. The culture was adjusted to OD$_{600}$=1.0, centrifuged for 15 min at 8 000 r.p.m., and then 100 µl PBS was added. Samples were sonicated until >95% of all cells were lysed (lysis was continuously monitored by phase-contrast microscopy) and 20 µl of each culture lysate containing 2× Laemmli sample buffer (Bio-Rad) with β-mercaptoethanol was analysed for the presence of Spo0A by Western blot analysis. A rabbit polyclonal anti-Spo0A antibody [32, 33], kindly provided by Dr. Masaya Fujita, was used at a 1:2 000 dilution.

For CPE Western blot, *C. perfringens* strains grown in 10 ml DS for 8 h at 37°C were adjusted to OD$_{600}$=1.0 and then sonicated until >95% of all cells were lysed (lysis was continuously monitored by phase-contrast microscopy). After sonication, each culture lysate was analysed for the presence of CPE by Western blot analyses using a rabbit polyclonal

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**Fig. 2.** Construction and characterization of *relA dtd* and *dtd* mutants of *C. perfringens* SM101. (a) Schematic diagram showing the Targetron insertion and primer target regions in wild-type strain SM101, *relA dtd* (strain RS102) and *dtd* (strain RS103) mutants. (b) RS102 and RS103 mutants and their complemented strains RS102(pRS3), RS102(pRS4), RS102(pRS11) and RS103(pRS11) were confirmed by amplification of the inserted group II intron (~2 kb) or *ermB* by colony PCR. The complemented strains were constructed using a *C. perfringens*-E. coli shuttle multicopy plasmid pJIR750. (c) The transcript levels of *relA* and *dtd* were measured using quantitative reverse transcriptase-PCR (qRT-PCR) with primers designed from the region downstream of the group II intron. The relative expression was calculated as relative to the wild-type after normalization to the housekeeping gene 16S rRNA. Results are represented as mean±SD of three independent experiments. Statistical significance was determined by one-way ANOVA compared to the wild-type. ***P<0.001, **P<0.01.
anti-CPE antibody as previously described [25]. IRDye 800CW infrared dye-conjugated secondary antibody (LI-COR) was used at a 1:10 000 dilution. The Odyssey LiCor CLx (LI-COR) was used to detect secondary antibody fluorescent emissions. The band intensities of the Western blots were quantified using ImageJ software (https://imagej.nih.gov/ij/).

**Phospholipase C activity measurement**

*C. perfringens* strains were grown overnight in FTG at 37 °C, and 200 µl of this culture was inoculated and propagated in 10 ml TGY for 5 h at the same temperature. After adjustment to OD<sub>600</sub>=1.0, 2.5 µl of the culture was spotted onto egg yolk agar plates. The inoculated plates were then incubated anaerobically for 24 h at 37 °C and translucent clearing zones were measured.

**Statistical analysis**

One-way ANOVA was used to determine statistical differences for comparisons. In all analyses, \( P<0.05 \) was considered significant.

**RESULTS**

**RelA and DTD homologues in *C. perfringens* strain SM101**

When we compared the *C. perfringens* SM101 relA locus with that of *B. subtilis* strain 168, the genetic arrangement of relA and dtd was found to be identical (Fig. S1a). Moreover, phylogenetic tree analysis of amino acid sequences of RelA and DTD revealed a high similarity between *Clostridium* species and *B. subtilis* 168 (Fig. S1b). The amino acid sequence of SM101 RelA [726 amino acids (aa), accession no. ABG86141] was found to have 53% identity to that of *B. subtilis* 168 (734 aa, accession no. NP_390638). Similar to *B. subtilis* 168 RelA, the SM101 RelA belonged to the RelA/ SpoT homologue family, and consists of a (p)pGpp hydrolase (HD) domain; a (p)pGpp synthetase (Syn) domain; a Thr-tRNA synthetase (ThrRS), GTPase and SpoT (TGS) domain; and an aspartate kinase, chorismate mutase and TyrA (ACT) domain (Fig. S2a). Motifs in HD and Syn domains, which are core functional domains in (p)pGpp metabolism, were completely identical in RelA from both strains. DTD similarity was also high between *Clostridium* species and *B. subtilis* 168 (Fig. S1b): the amino acid sequence of SM101 DTD (149 aa, accession no. ABG86731) was found to have 59% identity to that of *B. subtilis* 168 (146 aa, accession no. NP_390637) and the active site was in the same position of DTD in both strains (Fig. S2b). Therefore, we hypothesized that SM101 RelA-DTD might play a role in the stringent response, similar to *B. subtilis* 168.

**The expression of relA and dtd promoters in strain SM101**

Previous studies revealed that in *B. subtilis* 168, relA is part of a bicistronic operon together with the downstream gene dtd [34, 35]. To evaluate whether relA-dtd are organized as an operon in *C. perfringens*, the DNA upstream of the relA, dtd and CPR_1902 genes (Fig. 1a), which most likely contains the gene promoter, was fused to *E. coli* gusA, and GUS activity was measured after introducing the gusA-fusions into *C. perfringens* SM101. The vegetative culture of SM101 carrying relA-gusA fusion had some GUS activity. However, significantly higher levels of GUS activity were observed in sporulating cultures of SM101 carrying the relA-gusA fusion (Fig. 1b), strongly suggesting that a sporulation-specific promoter is located upstream of the relA locus. GUS expression from this relA promoter began after approximately 1 h of incubation in sporulation conditions, and reached a maximum after 4 h (Fig. 1b). In contrast, no detectable GUS activity was obtained from vegetative and sporulating cultures of SM101 carrying dtd-gusA fusion, indicating an absence of a promoter in the upstream region of the dtd locus. Furthermore, significant levels of GUS activity were obtained with both vegetative and sporulating cultures of SM101 carrying CPR_1902-gusA, strongly suggesting that a promoter is located upstream of CPR_1902. Also, since there is a rho-independent transcriptional terminator downstream from the dtd stop codon (data not shown), it is very unlikely that CPR_1902 is a part of the relA-dtd transcriptional unit. Collectively, these results indicate that relA and dtd are organized as a bicistronic operon and the relA promoter is strongly expressed during sporulation conditions.

**Construction and characterization of relA dtd mutants and their complemented strains**

To investigate the function of RelA in *C. perfringens*, we constructed relA (strain RS102) and dtd (strain RS103) mutants in SM101 using a TargeTron system (Fig. 2a). The identities of the mutants were confirmed by PCR analyses using cultures RS102 and RS103. Although 327 bp relA-specific or 526 bp dtd-specific PCR products were obtained with SM101 DNA, an approximately 2.5 kb product was obtained from RS102 and RS103, suggesting that group II intron-containing ermB was incorporated into wild-type relA in RS102 and wild-type dtd in RS103 (Fig. 2b). Finally, the amplification of 627 bp ermB-specific product from DNA of RS102 and RS103, but not from SM101, further supports the incorporation of ermB with a group II intron into the wild-type relA and dtd genes in RS102 and RS103, respectively (Fig. 2b).

As expected, relA transcript levels decreased significantly (\( P<0.001 \)) in relA mutant RS102 compared to wild-type SM101 (Fig. 2c). Moreover, dtd transcript levels were also decreased in RS102, suggesting that dtd is part of an operon with relA (Fig. 2c). Therefore, strain RS102 constitutes a double relA dtd mutant. Wild-type relA transcript levels and no dtd transcripts were detected in dtd mutant strain RS103 (Fig. 2c), suggesting RS103 is a single dtd mutant.

We also constructed relA- and dtd-complemented strains with their native promoter using *C. perfringens*-*E. coli* shuttle multicopy plasmid pIJR750 [31]. The relA dtd mutant RS102 was complemented with either the wild-type relA alone [pRS3; (P<sub>relA</sub>-relA)], the full relA-dtd operon [pRS4; (P<sub>relA</sub>-relA-dtd)] or dtd alone [pRS11; (P<sub>relA</sub>-dtd)] fused with...
the relA promoter (Table 1). Although relA transcript levels were restored in RS102(pRS3) and RS102(pRS4), the levels resulted in an approximately twofold increase in RS102 (pRS4) compared to the wild-type (Fig. 2c). As expected, relA levels in RS102(pRS11) were similar to those in relA dtd mutant RS102. In contrast, the relA levels in dtd mutant RS103 and its complemented strain RS103(pRS11) were similar to those of the wild-type. However, the dtd transcript levels in complemented strains RS102(pRS4), RS102 (pRS11) and RS103(pRS11) were higher than that in wild-type SM101 (Fig. 2c). dtd overexpression in complemented strains could be explained by the presence of multicopy dtd genes derived from multicopy plasmid vector pJR750 (Table 1). Collectively, relA and dtd were expressed in their respective complemented strains.

Disruption of relA leads to the slow growth of SM101

Previous studies demonstrated a growth defective phenotype of B. subtilis relA mutant [15, 18, 36]. When wild-type, mutant and complemented C. perfringens strains were grown in vegetative conditions for 12 h at 37 °C, the relA dtd double mutant exhibited a slow growth, which involved both prolongation of the exponential phase and a decrease in final cell density compared to the wild-type (Fig. 3). However, the growth of the dtd mutant was largely similar to that of the wild-type (Fig. 3). The growth defect in the relA dtd double mutant was restored to wild-type levels by complementing the relA dtd mutation with recombinant plasmids carrying the relA-dtd operon or relA alone, suggesting that the growth defect in the relA dtd double mutant was due to the loss of relA function. Further support came from the finding that the recombinant plasmid carrying dtd alone did not restore the growth defect of relA dtd. Collectively, our results indicate that the loss of RelA causes a phentotypic growth defect in relA dtd double mutants during vegetative conditions.

RelA and DTD are required for C. perfringens spore formation

Since a relA disruption in Bacillus species diminished or delayed spore formation [15, 19, 36], we next evaluated the spore-forming ability of C. perfringens relA dtd and dtd mutants. We found that spore-forming efficiency was decreased by >10^2-fold in both relA dtd (strain RS102) and dtd (strain RS103) mutants compared to the wild-type (P<0.001) (Table 2). The spore-forming efficiencies of complemented strains RS102(pRS3) and RS102(pRS11) were significantly lower than the wild-type, indicating that relA and dtd alone are not sufficient to rescue the spore formation defect in the relA dtd double mutant. However, wild-type level spore-forming efficiency was observed with RS102(pRS4), in which the relA dtd double mutant is complemented with the relA-dtd operon, indicating that the expression of both relA and dtd is required for spore formation. Further evidence for the requirement of dtd in spore formation came from the observation that strain RS103 (pRS11), in which the dtd mutant is complemented with dtd, had significantly higher frequency of spore formation than dtd mutant RS103, although it did not reach wild-type level. Collectively, these results indicate that both RelA and DTD are required for spore formation in C. perfringens strain SM101.

RelA and DTD positively regulate codY transcription

Previous studies with B. subtilis and C. perfringens showed that CodY positively regulates sporulation by activating early sporulation genes [15, 16]. Therefore, we hypothesized that the impaired spore formation of the relA dtd mutant strain may result from insufficient expression of codY. Thus, we analysed codY transcript levels in C. perfringens relA dtd and dtd mutants (Fig. 4a). The codY transcript level in the relA dtd mutant (strain RS102) was significantly decreased in 5 h DS cultures compared to the wild-type. The codY transcript was restored to wild-type levels when the
relA dtd mutant was complemented with a multicopy plasmid pJR750 carrying relA-dtd or dtd alone with a relA promoter. However, full rescue of codY level was not observed in a relA dtd mutant complemented with relA alone. These results indicate that RelA needs DTD to fully activate codY functions, but DTD in the absence of RelA is sufficient to activate codY expression. However, under similar growth conditions, decreased codY expression was observed in dtd mutant strain RS103 compared to the wild-type, and this defect was fully rescued when complemented with dtd alone, suggesting that dtd plays a minor role in activating codY expression in the presence of wild-type relA. Collectively, our results indicate that RelA and DTD activate transcription of codY during sporulation conditions.

### RelA/DTD positively regulate transcription of sporulation-specific genes

Because our results indicated that RelA/DTD activate codY expression in C. perfringens strain SM101, it is very likely that CodY enhances sporulation via activating the spo0A-dependent sporulation pathway [15, 16]. To explore this possibility, we evaluated the expression of spo0A (a master regulator of sporulation) using qRT-PCR and production of Spo0A using Western blot analysis. We also examined the transcript levels of spo0A-regulated σ factor genes, sigE, sigF, sigG and sigK [11].

spo0A expression was decreased by ~fourfold and ~threefold in relA dtd (strain RS102) and dtd (strain RS103) mutants, respectively, after a 5 h incubation in DS (Fig. 4b). Wild-type spo0A expression was restored in strain RS102(pRS4), in which the relA dtd mutant is complemented with the relA-dtd operon. However, spo0A expression was not fully restored in strains RS102(pRS3) (relA dtd mutant complemented with relA alone) or RS102(pRS11) (relA dtd mutant complemented with dtd alone). A partial restoration of spo0A expression was observed in RS103(pRS11), in which the dtd mutant is complemented with wild-type dtd. As expected, reduced levels of Spo0A production were observed in relA dtd mutant RS102 and dtd mutant RS103 after a 5 h incubation in DS. Interestingly, Spo0A production was restored in both RS102(pRS4) and RS103(pRS11), although lower spo0A transcript levels were observed in RS103(pRS11) compared to the wild-type (Fig. 4b). However, in consistence with spo0A expression results, no restoration of Spo0A production was observed in RS102(pRS3) or RS102(pRS11). These results indicate that both RelA and DTD are required to activate spo0A expression and Spo0A production.

A significantly lower transcript level was observed for all σ factors (sigE, sigF, sigG and sigK) in both RS102 and RS103 as RS102 compared to the wild-type (Fig. 4c). The expression of these σ factor genes was restored to wild-type levels in RS102(pRS4) and RS103(pRS11). However, σ factor gene expression was not fully restored in RS102(pRS3) or RS102(pRS11). These results indicate that both relA and dtd activate transcription of σ factor genes through upregulation of spo0A expression. In summary, our results indicate that RelA/DTD positively regulate transcription of Spo0A-dependent sporulation-specific genes.

### RelA/DTD positively regulate cpe expression and CPE production

Since C. perfringens strain SM101 produces CPE only during sporulation by activating sigK and/or sigE [9, 10, 37], it is very likely that loss of relA and dtd causes reduction of cpe expression and CPE production. Our results revealed a high level of cpe expression (relative expression 1.0) in wild-type SM101 following a 5 h DS incubation. However, cpe expression was reduced by <0.01 relative expression in 5 h DS cultures of relA dtd (strain RS102) and dtd (strain RS103) mutants (Fig. 5a). The cpe expression defects were fully restored to wild-type levels in complemented strains RS102(pRS4) and RS103(pRS11), but in RS102(pRS3) and RS102(pRS11), indicating that both relA and dtd are required for cpe expression (Fig. 5a).

As expected, almost no CPE production was observed in 8 h DS cultures of mutant strains RS102 and RS103 compared to wild-type SM101 (Fig. 5b). Loss of CPE production was fully restored in complemented strains RS102(pRS4), RS102(pRS11) and RS103(pRS11), but in RS102(pRS3), indicating that relA and dtd are required for CPE production.

### Table 2. Sporulation efficiency in C. perfringens strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotypes</th>
<th>Sporulation efficiency</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM101</td>
<td>Wild-type</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>RS102</td>
<td>relA·dtd</td>
<td>5.0×10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RS102(pRS3)</td>
<td>relA·dtd&lt;sup&gt;·&lt;/sup&gt;(P&lt;sub&gt;relA&lt;/sub&gt;-relA)</td>
<td>9.1×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RS102(pRS4)</td>
<td>relA·dtd&lt;sup&gt;·&lt;/sup&gt;(P&lt;sub&gt;relA&lt;/sub&gt;-relA-dtd)</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>RS102(pRS11)</td>
<td>relA·dtd&lt;sup&gt;·&lt;/sup&gt;(P&lt;sub&gt;relA&lt;/sub&gt;-dtd)</td>
<td>7.3×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RS103</td>
<td>dtd&lt;sup&gt;·&lt;/sup&gt;</td>
<td>4.3×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RS103(pRS11)</td>
<td>dtd&lt;sup&gt;·&lt;/sup&gt;(P&lt;sub&gt;relA&lt;/sub&gt;-dtd)</td>
<td>6.0×10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sporulation efficiency was determined based on the ratio of heat-resistant cells/total cells for a strain compared to wild-type. Data represents the average of three biological replicates.

<sup>b</sup> Statistical significance was determined by one-way ANOVA compared to the wild-type.

NS, not significant.
Fig. 4. Expression of codY, spo0A, sigma genes and production of Spo0A by C. perfringens strains. (a) RNA was extracted from indicated C. perfringens strains grown in DS for 5 h and qRT-PCR was performed for measuring expression of codY. Relative expression was calculated as relative to the wild-type after normalization to the housekeeping gene 16S rRNA. (b) Transcript level of spo0A (left panel) was analysed as in panel (a). Spo0A production (right upper panel) was determined in cell lysates prepared from C. perfringens strains grown in DS for 5 h by Western blot analyses using an anti-Spo0A antibody [32, 33] and the band intensities were quantified using ImageJ software (right lower panel). (c) Transcript levels of sigE, sigF, sigG and sigK were analysed as in panel (a). All data are represented as means±SD of three independent experiments. Statistical significance was determined by one-way ANOVA compared to the wild-type. ***P<0.001, **P<0.01, *P<0.05.
production. Interestingly, a higher level of CPE production was observed in complemented strains RS102(pRS4), RS102(pRS11) and RS103(pRS11) compared to the wild-type. This could be due to gene dosage effects, as complemented plasmids are the derivatives of a multicopy shuttle plasmid pIR750 (Table 1). Collectively, our results indicate that RelA/DTD positively regulate cpe expression and CPE production.

**RelA, but not DTD, negatively regulates PLC production**

All *C. perfringens* strains produce PLC, which is a major virulence factor for gas gangrene and histotoxic infections in humans [3]. Since *C. perfringens* can produce PLC during vegetative conditions [1, 2], we measured PLC production by spotting *C. perfringens* vegetatively grown cultures on egg yolk agar plates and measured clearing zones around the spotted cultures due to the phospholipase activity of PLC. After anaerobic incubation of culture spotted-egg yolk agar plates at 37 °C for 24 h, an increased translucent clearing zone was observed with *relA dtd* mutant strain RS102 compared to the wild-type SM101 (*P*<0.001) (Fig. 6a). The zone diameter of the *relA dtd* double mutant was rescued to nearly wild-type levels by complementing *relA dtd* mutant with *relA* alone [RS102(pRS3)] and the *relA-dtd* operon [RS102(pRS4)]; however, complementing with *dtd* alone [RS102(pRS11)] was not sufficient for rescue. Moreover, the zone diameters with *dtd* mutant strain RS103 and its complemented strain RS103(pRS11) were nearly similar to that of the wild-type (Fig. 6a). To confirm these phenotypic findings, we further analysed the transcript levels of *plc* using qRT-PCR. Consistent with phenotypic results, the *plc* expression was upregulated in *relA dtd* mutant RS102 compared to the wild-type and the *plc* expression was restored to wild-type levels in *relA dtd* mutant complemented with *relA* alone or *relA dtd* operon, indicating that *relA* negatively regulates *plc* expression. Furthermore, the *plc* expression levels in *dtd* mutant RS103 and its complemented strain RS103(pRS11) were nearly similar to that of the wild-type (Fig. 6b), indicating that *dtd* plays no role in *plc* expression. Taken together, these results indicate that RelA, but not DTD, negatively regulates PLC production in strain SM101 by downregulating *plc* transcription.

**DISCUSSION**

(p)ppGpp, which is mainly synthesized and degraded by RSH family protein RelA in furmicutes [38], coordinates bacterial physiology and virulence by regulating the cellular response to stressful conditions in many organisms [15, 39–41]. On the other hand, DTD prevents misincorporation of cytotoxic D-Tyr during protein synthesis by removing D-Tyr from tRNA^{D-Tyr} [42, 43]. The molecular machineries related to a stringent response vary widely among bacterial species [38, 44] and have not been studied in the important human intestinal pathogen *C. perfringens*; therefore, we characterized *C. perfringens* relA-dtd locus by expression analyses and gene knock-out studies.

The first significant finding of our study is that *relA* and *dtd* forms a bicistronic operon (Fig. 1) as seen in *B. subtilis* [34, 35]. Our gusA assay identified promoter activity upstream of *relA* and CPR_1902, but not of *dtd*, indicating that *relA* and *dtd* are transcribed from a single promoter located upstream of *relA*. Our qRT-PCR analyses confirmed our *gusA* assay results by showing that insertion of a TargeTron in the *relA* locus hampered the transcription of *relA* as well as
the downstream dtd gene (Fig. 2c). Furthermore, identification of relA and dtd transcripts in wild-type strain SM101 indicated that both relA and dtd are functional, as opposed to B. subtilis strain 168 in which DTD lacks 24 residues from its N-terminal domain [23]. relA promoter activity during both vegetative and sporulation conditions also suggests that RelA and DTD might control cell physiology through the regulation of (p)ppGpp and t-RNA^Tyr^ levels, respectively, during vegetative and sporulation growth of C. perfringens.

The second significant finding is that loss of RelA causes a phenotypic slow growth during vegetative conditions (Fig. 3), suggesting that increased (p)ppGpp levels may result in the slow growth of SM101, as seen in B. subtilis [15, 18, 36]. In B. subtilis strain 168, the inactivation of relA alone [lack of (p)ppGpp hydrolase activity] is known to increase cellular (p)ppGpp levels, since this strain has two other proteins, YwaC and YjbM, with (p)ppGpp synthetase activity [18, 36, 45, 46]. Moreover, the activity of the hydrolase domain of RelA is required for maintaining the basic and efficient growth of cells because without its hydrolase function, even a small amount of (p)ppGpp can reach levels that are toxic for bacterial growth [18]. In the current study, we demonstrated the presence of a YjbM homologue CPR_0621 in C. perfringens SM101 by showing the following: (1) CPR_0621 (263 aa, accession no. ABG85819) belongs to the RSH family, which is assumed to act as a (p)ppGpp synthetase in C. perfringens strain SM101; (2) CPR_0621 had 57 and 39% amino acid sequence identity with YjbM and YwaC, respectively, and Syn domain motifs are identical in CPR_0621 and YjbM (Fig. S3a); (3) phylogenetic tree analysis of Syn domains reveal CPR_0621 as a homologue of YjbM (Fig. S3b); and (4) CPR_0621 is expressed in SM101 and isogenic relA dtd mutant, as CPR_0621 transcript levels were similar between wild-type SM101 and relA dtd mutant strain RS102 (P>0.05; Fig. S3c). Collectively, these results show the possibility that, although CPR_0621 functions as a (p)ppGpp synthetase in both SM101 and RS102, relA-disrupted strain RS102 (lacking (p)ppGpp hydrolase activity) acquired both increased (p)ppGpp levels and the (p)ppGpp-induced depression of GTP pool compared to SM101, which leads to the slow growth of RS102 during vegetative conditions as previously described for B. subtilis [18]. Further functional analysis of CPR_0621 should provide additional insight into its physiological role.

The third significant finding of the current study is that both RelA and DTD are required for spore formation by C. perfringens. Our finding that RelA is required for spore formation by C. perfringens is consistent with the results obtained previously with B. subtilis strain 168 [14, 15, 19]. However, our finding that DTD is also required for C. perfringens spore formation is novel in that no such role of DTD was demonstrated in B. subtilis 168, as this strain has a truncated DTD lacking 24 N-terminal residues [23]. The reason for the lack of full rescue of the spore formation defect in RS103(pRS11) (dtd mutant complemented with wild-type dtd) is not clear. One possibility is that t-RNA^Tyr^ as well as (p)ppGpp may function optimally in sporulation within a narrow concentration range.

The fourth significant finding of our study is that RelA/DTD positively regulates spore formation and CPE production by Spo0A-dependent activation in response to CodY.
regulation. Previous studies with \emph{C. perfringens} SM101 have demonstrated that Spo0A and Spo0A-specific \( \sigma \) factors (\( \sigma^E \), \( \sigma^F \), \( \sigma^G \) and \( \sigma^K \)) are required for spore formation and CPE production \cite{8-11}. Our findings from transcriptional analyses suggest that the impaired spore formation and CPE production of \textit{relA} \textit{dtd} and \textit{dtd} mutants resulted from insufficient expression of \textit{codY}. The expression of \textit{codY}, \textit{codY}-regulated sporulation-specific \textit{spo0A}, and \textit{sigE}, \textit{F}, \textit{G} and \textit{K} genes were significantly repressed in \textit{relA} \textit{dtd} and \textit{dtd} mutants, and expression was restored in complemented strains. Our results coupled with previous results \cite{8-11, 16} demonstrate that \textit{RelA}/\textit{DTD} positively regulates spore formation and CPE production by activating \textit{CodY}-regulated sporulation-specific factors. However, further study will be needed to clarify the interaction between \textit{RelA}/\textit{DTD} and \textit{CodY}.

Finally, we found that \textit{RelA} negatively regulates PLC production by repressing \textit{plc} expression. However, we could not validate \textit{RelA}-dependent signalling pathways in PLC production. The \textit{relA} \textit{dtd} double mutant, but not the \textit{dtd} single mutant, produces higher levels of PLC protein and \textit{plc} transcripts compared to the wild-type during vegetative growth. The lack of PLC overproduction in the \textit{dtd} mutant cannot be explained by the growth defect as the \textit{dtd} mutant grew almost like wild-type in rich TGY medium (Fig. 3). PLC production and \textit{plc} expression were restored to nearly wild-type levels by complementation of the \textit{relA} \textit{dtd} mutant with \textit{relA} alone and the \textit{relA}-\textit{dtd} operon, but not with \textit{dtd} alone. These results indicate that the overproduction of PLC was due to the loss of \textit{RelA} alone. As \textit{CodY} did not affect PLC production in a \textit{C. perfringens} type \textit{D} strain \cite{12}, it is very unlikely that \textit{RelA}-mediated PLC repression occurs through \textit{CodY} regulation. However, it is possible that \textit{RelA} negatively regulates \textit{plc} expression and PLC production by repressing positive regulator(s) of PLC, such as VirR/VirS, \textit{agr}, VR-RNA and \textit{VirX} \cite{47-50}. Further studies on the evaluation of expression of these positive activators in the \textit{relA} \textit{dtd} double mutants would further our understanding of the possible cross-talk between \textit{RelA} and toxin-regulatory systems.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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