

Title: Deletion of *rgaS* reduces virulence in a mouse model of *Clostridioides difficile* infection.

Running title:

Authors

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Highlights

- The gene encoding histidine kinase *rgaS* (R20291_0503) was deleted in *C. difficile* R20291.
- Cytotoxicity towards Vero cells was reduced for the *rgaS* mutant signifying a reduction in TcdA and TcdB in the supernatant.
- Western blot analysis revealed a reduction in the amount of CDT in the supernatant of the *rgaS* mutant.
- The mutant was less virulent in a mouse model of *C. difficile* infection, compared with the wildtype parental.
- These data demonstrate the contribution of *RgaS* to *C. difficile* virulence.

Keywords:

Abstract

Two-component system signalling is involved in several processes relevant to *Clostridioides difficile* virulence. We demonstrate that deletion of the orphan histidine kinase *rgaS* (R20291_0503), reduces detectable toxin in the supernatant of R20291 and impairs virulence in a mouse model of infection. These data suggest that *RgaS* regulates virulence capabilities in hypervirulent *C. difficile*.

Short communication

Considered an urgent threat by the Centers for Disease Control and Prevention, *Clostridioides difficile* (formerly *Clostridium difficile* [1]) is the leading cause of hospital-associated diarrhoea in developed nations and poses a significant burden to healthcare systems. Estimated annual costs exceed \$5b in the USA alone [2]. The main virulence

factors responsible for eliciting disease symptoms ranging from self-limiting diarrhoea through to the potentially fatal pseudomembranous colitis, are the monoglucosyltransferases Toxin A (TcdA), and Toxin B (TcdB) [3]. Hypervirulent strains, in particular those belonging to NAP1/B1/027, PCR ribotype 027 (RT 027) produce a third toxin, the ADP-ribosylating binary toxin, or *C. difficile* transferase (CDT). These strains have been responsible for outbreaks and severe cases of disease [4]. CDT enhances the virulence of TcdA and TcdB-producing strains in rodent models of *C. difficile* infection (CDI) [5-7] and the presence of detectable CDT in patient stool is associated with worse incidence of disease [8].

Bacterial two-component system (TCS) signalling has been shown to regulate many fundamental processes to *C. difficile* biology and virulence including sporulation, flagellum and toxin production [10]. Bacterial TCSs typically comprise a transmembrane sensory histidine kinase (HK) which autophosphorylates in response to an environmental stimulus and its cognate response regulator (RR), to which the phosphoryl group is transferred. Phosphotransfer from HK to RR leads to conformational changes in the RR, usually leading to homodimerization which then allows for transcriptional regulation of target genes through promoter binding [9]. Fifty-two proteins annotated as histidine kinases are encoded on the chromosome of the archetypal RT 027 strain, R20291.

While most characterised TCS genes are located in tandem on the chromosome, with HK and RR present at the same locus, the so-called “orphan” HKs are situated in the absence of an adjacent RR gene. One such orphan histidine kinase in *C. difficile* is the recently characterised RgaS. Knockdown and gene deletion of the 630 homolog (CD0576), has been shown to reduce sporulation frequency [11]. Owing to sequence similarities

between CD0576 and VirS in *Clostridium perfringens*, a VirR homolog was identified (CD3255) which was found to be orphan in nature and was also demonstrated to affect sporulation after gene knockout in 630 and R20291 strain backgrounds. The authors stipulated that these proteins coined RgaS and RgaR, function as a cognate TCS [11].

Despite the involvement of VirR/VirS in the regulation of toxin production in *C. perfringens* [12], the contribution of RgaS to virulence has not yet been determined in *C. difficile* and is the focus of this study.

To this end, we created a deletion mutant of *rgaS* (R20291_0503) in the CRG2021 lineage of R20291 [13] using allelic-exchange technology exactly as described before [14, 15] but with primers specific to *rgaS*. The deletion strain is designated *R20291ΔrgaS* (Fig. S1a). All strains and plasmid information are provided in Table S1 and primer details are provided in Table S2, along with detailed methodologies in the supplementary materials.

To ensure that any ensuing phenotypic differences are ascribed to the deletion of *rgaS*, we attempted to complement the *rgaS* gene at the *pyrE* locus under the transcriptional control of its native promoter. Unfortunately, it was not possible to clone this construct without unintended mutations, demonstrating that the product was toxic to the *E. coli* cloning host. To circumvent this encountered toxicity, we placed the *rgaS* gene under the control of the P_{thlOID} promoter, comprising the strong constitutive *Pthl* promoter from *Clostridium acetobutylicum*, adapted to contain a *lacO* operator sequence from the *E. coli lac* operon, to reduce expression during cloning (Fig. S2/ Data S1)[16]. Moreover, we integrated the *lac* repressor (*lacI^q*) onto the complementation plasmid Fig. S2/Data S2. Using this approach in combination with the XL-1 Blue *E. coli* cloning host containing an episomal copy of *lacI^q*, we successfully cloned this construct into pMTL-YN2C [15] and

thereafter complemented *rgaS* at the *pyrE* locus. The resultant complementation strain is designated R20291 Δ *rgaS* *P_{thIOID}-*rgaS* (Fig. S1b).

We first assessed any impact of gene deletion on sporulation. In the first report describing the manipulation of *rgaS*, deletion thereof led to a 1.6-fold reduction in sporulation frequency in R20291 [11]. The *rgaS* gene was also identified as being essential for sporulation in a genome-wide TraDIS experiment in R20291 [17]. We assessed sporulation in 120h cultures of our *rgaS* mutants. No discernible difference was observed between the deletion mutant and the wildtype parental (Fig. 1a). However, it must be noted that the assay adopted here is less sensitive than those applied in the aforementioned article [11]. To our surprise, the complemented strain was completely devoid of detectable spores. We hypothesise that the overexpression of *rgaS* using the constitutive P_{thIOID} promoter may interfere with signalling cascades that lead to spore formation. No difference in growth characteristics was observed during a 24-hour growth curve experiment between the three strains (Fig. 1b).

To determine whether deletion of *rgaS* affects *C. difficile* virulence, we assessed toxin production of the three strains using *in vitro* techniques. At the 48h timepoint, the deletion mutant produced less CDTa as determined by Western blot analysis (Fig. 2a). Ponceau staining confirmed that the reduction in CDTa observed was not a consequence of lower protein loading into that well. CDTa production was partially restored by complementation of *rgaS* at the *pyrE* locus. To determine any differences in TcdA and TcdB production, we assessed the virulence of each strain using an established cytotoxicity assay by treating monolayers of Vero cells with cell-free supernatants collected at the 48h timepoint as previously described [5, 14, 18]. We observed a more

than 10-fold decrease in cytotoxicity in the *rgaS*-deletion mutant compared with the wildtype parental which was partially restored by complementation at the *pyrE* locus (Fig. 2b). These results demonstrate that *rgaS* contributes to the virulence potential of *C. difficile* R20291. The precise mechanism has not been elucidated here. Although the supernatants contained less toxin, this could be a consequence of reduced transcription of toxin genes leading to reduced toxin production. Alternatively, this phenotype might be a consequence of reduced toxin export from the cell. Mechanistically, this reduction in detected toxin may be a consequence of impacted RgaR activation, thus reducing the transcription of its target genes and regulatory RNAs that have already been characterised [11]. On the other hand, it is possible that RgaS interacts with other RRs for which a relationship has not yet been reported in the literature.

To substantiate the involvement of *RgaS* in *C. difficile* virulence, we assessed each strain using a lethal murine model of CDI [6, 7]. For each infection group, ten individual mice were challenged with 5×10^3 spores. Since the complemented strain is devoid of spore-forming capabilities, only the wildtype and deletion mutant were assessed. Two independent experiments were performed, representing 40 animals altogether. After monitoring for clinical symptoms of infection across both experiments, in the groups infected with the wildtype parental, 16/20 animals reached experimental endpoint by the fourth day of infection, with only 4/20 surviving until the experimental endpoint. (Fig. 3). In contrast, 14/20 of the animals challenged with *R20291ΔrgaS* survived until the end of the experiment. The difference in time to endpoint between groups infected by each strain, was statistically significant ($p=0.0008$). This observation corroborates the data generated from the *in vitro* experiments and demonstrates that *RgaS* contributes to

virulence in R20291, presumably a consequence of reduced toxin production or possibly toxin export.

The mechanism by which RgaS affects virulence has not been elucidated. CDT production is regulated by the orphan RR CdtR, which is activated by phosphorylation of Asp61 [19]. CdtR also regulates the production of TcdA and TcdB in RT 027 strains. Inactivation thereof by ClosTron mutagenesis [20] and introduction of a 69bp deletion within *cdtR* [21], attenuates virulence in rodent models of CDI. The TCS HK(s) which activate CdtR through phosphorylation, have not yet been identified. One potential hypothesis is that the orphan HK RgaS, activates the orphan RR CdtR, through TCS signalling. To test this hypothesis, we attempted to verify a protein-protein interaction using the bacterial two hybrid system (BACTH) [22, 23]. Unfortunately, we were unable to test for interaction between the two proteins since it was not possible to clone the *rgaS* construct into either pKT25 or pUT18 BACTH plasmids. This was unsurprising given the observed toxicities when attempting to clone *rgaS* into pMTL-YN2C for complementation in R20291Δ*rgaS*. Investigating protein-protein interactions between RgaS and CdtR, and between RgaS and RgaR, are important considerations for future studies.

We conducted a NCBI BLASTn search to determine whether *rgaS* is conserved across multiple RTs including those that possess or lack, the *cdtR* gene. Analysis revealed 269 matches for *C. difficile* sequences with ≥95% nucleotide sequence identity to the gene in R20291 (data not shown). Analyses included the hypervirulent RT 027 strains UK1 and CD196 (*cdtR*⁺, 100% ID *rgaS*), the RT 012 strain 630 (*cdtR*⁺, 99.48% ID *rgaS*), the hyper toxin-producing RT 087 strain VPI 10643 (*cdtR*⁺, 99.40% ID *rgaS*), as well as hypervirulent RT 078 strain M120 (95.53% ID *rgaS*) whose CdtR is non-functional [19]. Interestingly, *rgaS*

is also conserved in the RT 017 strain MAR286 which lacks a *cdtR* gene altogether (98.51% ID *rgaS*). The presence of the *rgaS* gene is therefore not associated with the presence/absence of *cdtR*.

In this study, we demonstrate that the orphan histidine kinase *RgaS* affects *in vitro* toxin production (or export) which corresponds to altered virulence in a mouse model of CDI. Our results provide supporting evidence of the importance of TCS signalling in the virulence of *C. difficile*.

Acknowledgements

Figure 3 (panel a) was made using [Biorender.com](https://biorender.com).

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182 Visualization: TWB

183 Writing – original draft preparation: TWB, MES

184 Writing – review and editing: TWB, MES, NH, WAP, SAK, NPM

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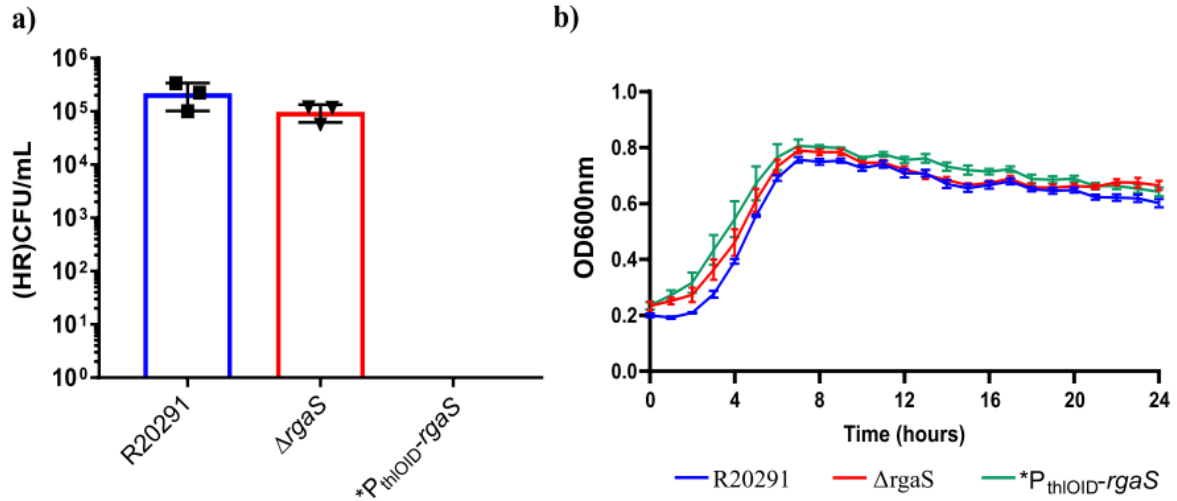
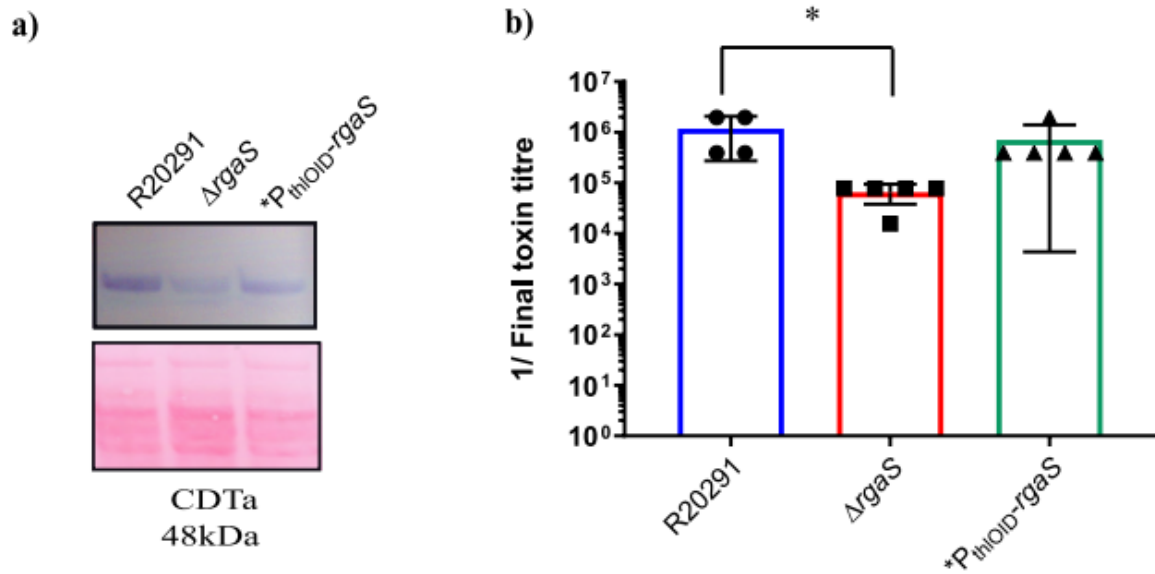


Figure 1 Effects of *rgaS* mutation on growth and sporulation.

b) Spore assay for R20291 and the *rgaS* mutants. Enumeration of HR-CFU (heat-resistant) following heat treatment at 65°C for 30 mins. Data represent the mean $\pm$ SD of ≥ 3 replicate cultures. No statistically significant differences were observed between R20291 and R20291 $\Delta rgaS$ ($P=0.1439$) as determined by one-way ANOVA according to Dunnett's multiple comparison test; **b)** Automated 24h growth curve comparing the growth characteristics of R20291 and the two *rgaS* mutants. Datapoints represent the mean $\pm$ SD of ≥ 3 replicate cultures.



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273 **Figure 2: *rgaS* mutants are affected for *in vitro* toxin production. a)** Detection of CDTa
 274 from cell-free supernatants collected at 48h. Upper panel represents the Western blots
 275 developed with a polyclonal antibody for CDT. Lower panel represents ponceau loading
 276 control stain. **b)** Cytotoxicity of 48h supernatants against monolayers of Vero cells. Data
 277 represent the mean $\pm$ SD of ≥ 4 replicate cultures. $P=0.043$ as determined by one-way
 278 ANOVA according to Dunnett's multiple comparison test.

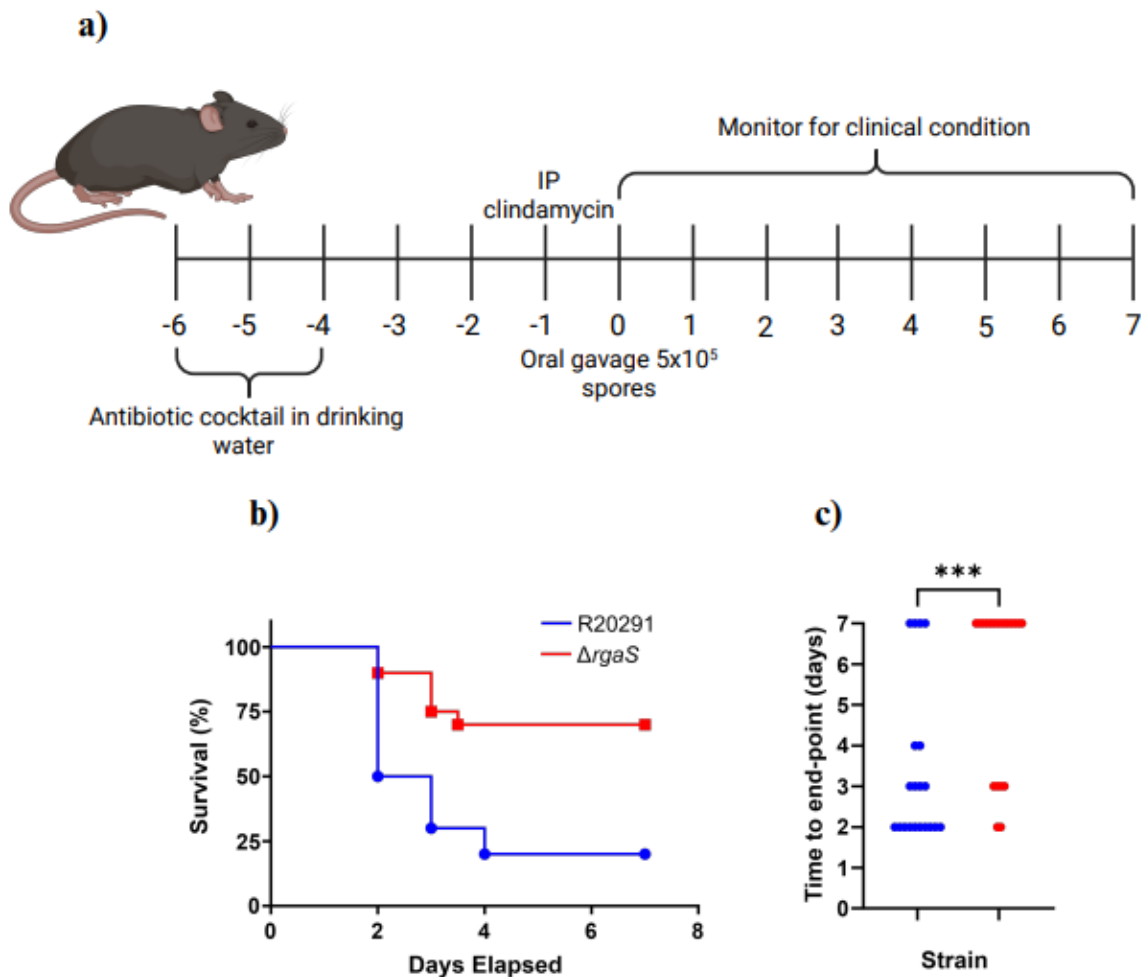


Figure 3: Deletion of *rgaS* reduces virulence in a mouse model of CDI. Antibiotic-conditioned male C57BL/6J mice were challenged with 5×10^3 spores of wildtype R20291 (n=20) and R20291 $\Delta rgaS$ (n=20) and were monitored for clinical symptoms of infection using a scoring system over a seven-day period. Mice were terminated when their clinical score reached or exceeded 14, or at the end of the experiment. **a)** Schematic of the mouse experiment. IP, intraperitoneal injection. **b)** Survival data expressed as % survival. Survival curves are significantly different ($P = 0.0011$) according to the Log-rank (Mantel-Cox) test; **c)** Time to end-point for each animal subject. $P = < 0.0008$ as determined by unpaired two-tailed T-test. Data are pooled from two independent experiments with 10 animal subjects per infection group, per experiment.