

Supplementary information

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

Authors

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Methodology

Microbiological culture

E. coli cultures were grown on LB medium at 37°C. Liquid LB and solid LB were supplemented with 25µg/ml chloramphenicol (Cm), where necessary. *C. difficile* was maintained on Brain heart infusion medium (Oxoid) supplemented with 5µg/ml yeast extract, 0.1% w/v L-cysteine (BHIS), where necessary 250µg/ml D-cycloserine, and 8 µg/ml cefoxitin (BHIS CC), and 15 µg/ml thiamphenicol (BHIS CCTM). *C. difficile* cultures were grown in an anaerobic workstation (Don Whitley) under anaerobic conditions comprising 80% N₂, 10% H₂ and 10% CO₂ gas atmosphere.

For phenotypic assays including automated growth curve analysis, cell cytotoxicity and toxin detection by Western blot, *C. difficile* strains were cultured overnight on BHIS CC plates. Individual colonies of each strain were subsequently inoculated into 1ml of BHIS for 4-6h, representing the experimental replicates. Dilution series were derived from the resultant cultures in fresh BHIS medium and incubated overnight. The following morning, the 10⁻³ dilution of each replicate was inoculated into sterile pre-reduced, pre-warmed tryptose-yeast extract (TY) medium to a final concentration of 1:1000. This resulted in consistent starting optical density OD_{600nm} (OD) values. TY medium was adopted for all phenotypic assays.

Generation of *rgaS* deletion and complementation mutants in R20291

The *rgaS* gene was deleted in R20291Δ*pyrE* using plasmid pMTL-YN4-*rgaS*. To obtain this plasmid, the homology arms for the deletion of *rgaS* by allelic-exchange technology were amplified using *rgaS* LHA F/ *rgaS* LHA R and *rgaS* RHA F/ *rgaS* RHA R primer sets. The two homology arms were spliced together by SOEing PCR, and the resultant fragment was cloned into pMTL-YN4 following restriction digestion with SbfI and AscI

and subsequent ligation. The resultant plasmid was confirmed to be as intended by Sanger sequencing of the insert region.

pMTL-YN4- *rgaS* was conjugated into R20291 Δ *pyrE* and single crossover integrants were isolated exactly as described previously [1-3]. Thereafter, double crossover recombinants in which the plasmid had excised along with the wildtype *rgaS* locus were isolated exactly as described previously and were confirmed to be deletion mutants using *rgaS* diag F/*rgaS* diag R primers (Fig S1). The amplicon was confirmed to be as intended by Sanger sequencing. The *pyrE* locus was then restored to wildtype exactly as described previously [1-3].

To generate the complementation vector, the *lacI^q* construct was cloned into pMTL-YN2C as an SbfI-SbfI fragment. Thereafter, the P_{thlOID} promoter was fused to the *rgaS* gene by SOEing PCR using P_{thlOID}-*rgaS* LHA F/ P_{thlOID}-*rgaS* LHA R and P_{thlOID}-*rgaS* RHA F/ P_{thlOID}-*rgaS* RHA F, primer sets. The resultant NotI-BamHI fragment was cloned into pMTL-YN2C following restriction digestion and ligation. Both insert sequences were confirmed by Sanger sequencing. The sequence of both fragments in FASTA format, is provided in Data S1 and Data S2. Complementation concomitant with *pyrE*-restoration was performed as described previously [1-3] and confirmed by PCR with *pyrE* WT F and P_{thlOID}-*rgaS* RHA R primers (Fig. S1b). The amplicon was confirmed to be as intended by Sanger sequencing.

Automated 24h growth curve

Growth characteristics of the three strains was assessed by automated growth curve exactly as described previously using a Promega Glomax plate reader [4].

Western blot detection of *C. difficile* toxins

Production of CDTa was assessed by Western blot on 48h cell-free supernatants using an HRP-Chicken anti-*Clostridium difficile* Binary Toxin Subunit A antibody (Gallus Immunotech), exactly as described previously [1].

***In vitro* cytotoxicity assay**

Cytotoxicity towards monolayers of Vero cell lines was assessed using an *in vitro* cytotoxicity assay exactly as described previously [1]. The end point (1/final toxin titre) was determined for each replicate as the greatest dilution at which ≥50% of the cells had rounded.

Sporulation assay

Individual 20mL cultures were prepared for each replicate in TY medium, as described in the Microbiological Culture section. At the 120h time-point, 1mL of sample was collected in a microfuge tube for each replicate and heat-treated at 65°C for 30m to destroy vegetative cells. Serial dilutions were prepared from the heated samples and three 20uL volumes were individually spotted onto BHIS CC plates containing 0.1% (w/v) taurocholate to promote spore germination. Plates were incubated anaerobically for 48h before calculating the number of heat-resistant colony-forming units per mL of sample (HR-CFU/mL), representing the number of recoverable spores.

Mouse model of CDI infection

Experiments were carried out using 8- to 12-week-old male C57BL/6J mice from the Jackson Laboratory. All animals were housed under specific pathogen-free conditions at the University of Virginia's animal facility. All procedures were approved by the

Institutional Animal Care and Use Committee at the University of Virginia under protocol number 3769-05-19. The protocol was approved on 11/19/2019. Mice were infected using a previously established murine model for *C. difficile* infection [5]. Six days before infection, mice were given an antibiotic cocktail in drinking water consisting of 45 mg/L vancomycin (Mylan, Canonsburg, PA), 35 mg/L colistin (Sigma, St. Louis, MO), 35 mg/L gentamicin (Sigma, St. Louis, MO), and 215 mg/L metronidazole (Hospira, Lake Forest, IL). Three days later, mice were switched to regular drinking water for 2 days, and the day before infection, they were given a single intraperitoneal injection of 0.016 mg/g clindamycin (Pfizer, New York, NY). The day of infection, mice were orally gavaged with 5×10^3 spores of *C. difficile* strains as indicated (R20291 and R20291 Δ rgaS). Mice were monitored daily during the course of infection and twice daily during the acute phase (days 2 and 3). Mice were immediately euthanized after the development of severe illness as measured by clinical scoring parameters. These parameters included weight loss, coat condition, eye condition, activity level, posture, and diarrhea, which were evaluated and assigned a numerical score and then added together to give a final clinical score between 0 and 20. Severe disease was indicated by a clinical score of 14 or higher, and any mouse scoring at or above that cutoff was immediately euthanized.

Table S1: Strains and plasmids used in this study

Strain/Plasmid	Description	Reference/Source
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Strain***E. coli***

XL-1 Blue	lacI ^q -positive cloning host	Stratagene, USA
CA434	Conjugal donor	[6]

C. difficile

R209291	Wildtype RT 027 isolate. CRG2021 lineage.	[7]
R209291 Δ <i>pyrE</i>	<i>pyrE</i> mutant for allelic exchange mutagenesis	[8]
R209291 Δ <i>pyrE</i> Δ <i>rgaS</i>	As above with <i>rgaS</i> deleted.	This study
R209291 Δ <i>rgaS</i>	<i>pyrE</i> -restored <i>rgaS</i> - deletion mutant	This study
R209291 Δ <i>rgaS</i> *P _{thlOID} - <i>rgaS</i>	<i>rgaS</i> -deletion mutant with <i>rgaS</i> complemented at the <i>pyrE</i> locus under the transcriptional control of PthlOID.	This study

Plasmid

pMTL-YN4	Knock-out vector for R20291	[8]
pMTL-YN4- <i>rgaS</i>	Knock-out vector for <i>rgaS</i> in R20291	This study
pMTL-YN2	<i>pyrE</i> -restoration vector for R20291	[8]
pMTL-YN2C	Complementation vector for R20291	[8]
pMTL-YN2C-P _{thlO1D} - <i>rgaS</i> (<i>lacI^q</i>)	Complementation of <i>rgaS</i> at the <i>pyrE</i> locus of R20291Δ <i>rgaS</i>	This study

Table S2: Primers used in this study

Primer	Sequence 5'-3'
<i>rgaS</i> deletion	
<i>rgaS</i> LHA F	TTTTTCCTGCAGGCAATGCTTTTGTATTATTCCATTC
<i>rgaS</i> LHA R	TTTAACTACCTCTACACGTGTAGTAAATAGATTGT
<i>rgaS</i> RHA F	ACGTGTAGAGGTAGTTAAAATTGCTATTCCACTTAG
<i>rgaS</i> RHA R	TTTTTGCGCGCCCTTCTAACCTTAAAAGTTTCTAATAC
<i>rgaS</i> diag F	CAATATAAGTACGTATTTTGTCTAAC
<i>rgaS</i> diaf R	GAAGTTACATCTTTAAAGAGCATTTC

Single crossover (SCO)

determination

YN4 F	CTCCATCAAGAAGAGCGAC
YN4 R	CTTATCCAGGGTGCTATC

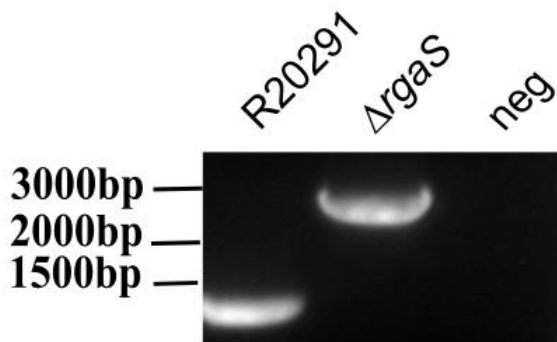
pyrE restoration

<i>pyrE</i> WT F	GGAGCTACTTGTATCCAAG
<i>pyrE</i> WT R	CCTAATTCCTTGAACCTC

rgaS complementation

P _{thlOID} - <i>rgaS</i> LHA F	TTTTTGCGGCCGCCACTAGTTTTTTAACAAAATATATTG
P _{thlOID} - <i>rgaS</i> LHA R	ACTTTATCATGAACTAACCTCCTAAATTTTG
P _{thlOID} - <i>rgaS</i> RHA F	AGGTTAGTTCATGATAAAGTCAAGCTTATTTTG
P _{thlOID} - <i>rgaS</i> RHA R	TTAAATCACATGATTAAGTCTTTC
<i>lacI</i> ^q F	TTTTTCCTGCAGGAAACGACACCATCGAATGGTG
<i>lacI</i> ^q R	TTTTTCCTGCAGGAACTTAATTGCGTTGCGCTCACTGCC

a)



b)

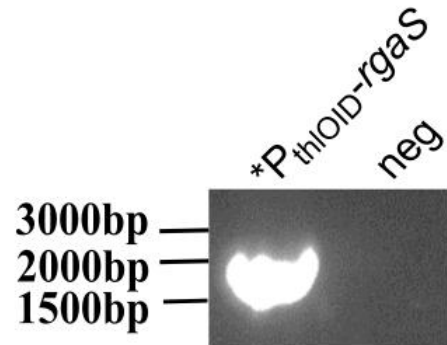


Figure S1: Authentication of *rgaS* mutant strains. **a)** Authentication of *rgaS*-deletion mutant by PCR. The region encompassing the *rgaS* locus was amplified using *rgaS* diag F/ *rgaS* diag R primers by colony PCR. **b)** Authentication of the *rgaS* complemented mutant by colony PCR using *pyrE* WT F and *rgaS* RHA R primers. All amplicons were separated by electrophoresis on a 0.75% agar gel, alongside a 1kbp plus ladder (NEB). Neg = negative control with no DNA template included.

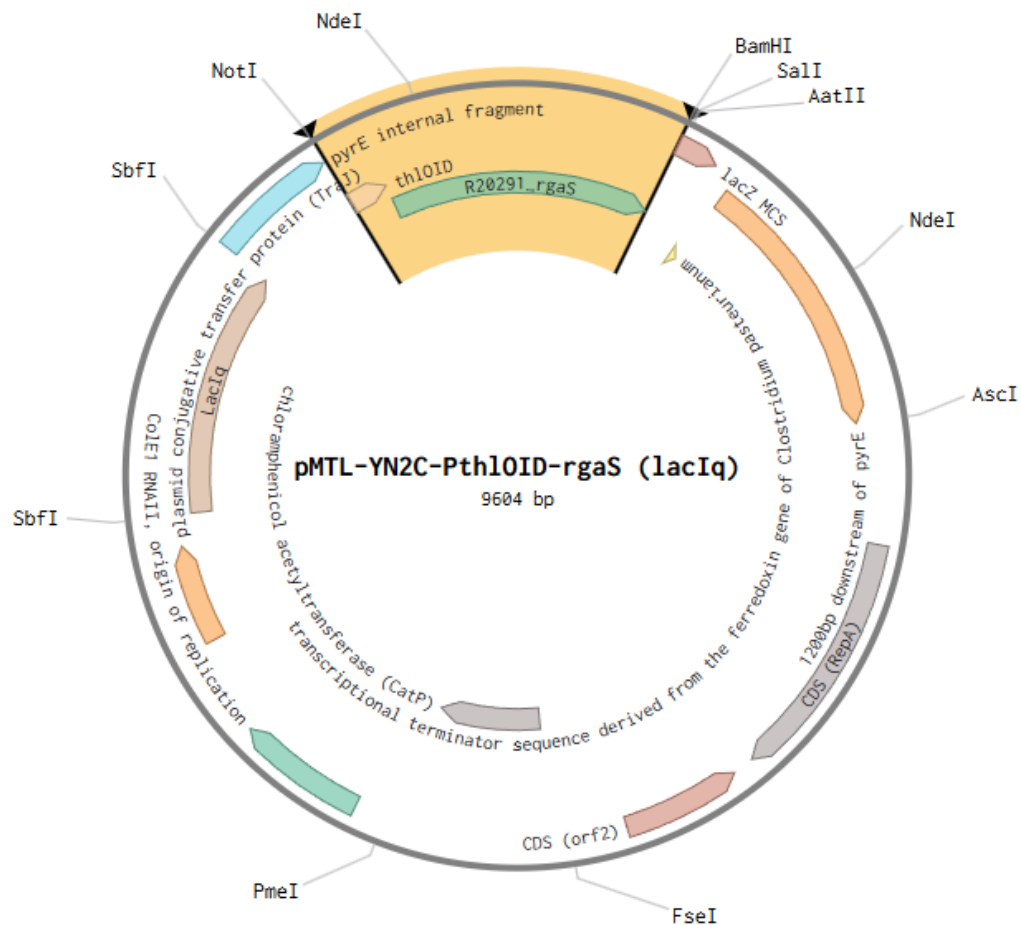


Figure S2: Plasmid map of pMTL-YN2C-Pth10ID- *rgaS* (*lacI^q*). Plasmid map of vector for complementation of *rgaS* under the transcriptional control of P_{thl01D} at the *pyrE* locus of R20291 Δ *rgaS*. Bespoke Pth10ID-*rgaS* insert highlighted as a NotI-BamHI fragment. Plasmid map prepared in using Benchling (<https://benchling.com>).

Data S1: FASTA DNA sequence of pMTL-YN2C-PthlOID-*rgaS* (lacIq) NotI-PthlOID-*rgaS*

BamHI insert

gcggccgccactagttttttaacaaaatatattgataaaaataataatag
tgggtataattaatgtggaattgtgagcgctcacaattggtgtgttgta
gagaaaacgtataaattagggataaactatggaacttatgaaatagattg
aaatggttttatctgtgctagtgatatcaaaatttaggaggttagttcatga
taaagtcaagcttatttttgaatatatttcattgcgatatcctttatgata
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ataaaattgatagtggaatagagttatttgtgttaaaggtgatggtatg
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tactaaaaatggagatatTTTtgacagacaaaaaaggTtctcttcatggac
ttggaataaaaagtgtaaaaaactctgtaaataagtatggTggtaatgtt
gtaattgaatatgatgataaaaaatttgtagttaaaattgctattccact
tagaaatgtgaagaaagcagttaatcatgtgatttaaggatcc

Data S2: FASTA DNA sequence of pMTL-YN2C-PthlOID-*rgaS* (lacIq) *Sbfl-LacI^q*-*Sbfl* insert

cctgcaggaaacgacaccatcgaatggtgcaaacctttcgcggtatggc
atgatagcgcccggaagagagtcaattcaggggtggtgaatgtgaaaccag
taacggtatatacgatgtcgcagagtatgccggtgtctcttatcagaccgtt
tcccgcgtggtgaaccaggccagccacgtttctgcgaaaacgcgggaaaa
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ctggccctgcacgcgcgctcgcaaattgtcgcggcgattaaatctcgcgc
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tcgaagcctgtaaagcggcggtgcacaatcttctcgcgcaacgcgtcagt
gggctgatcattaactatccgctggatgaccaggatgccattgctgtgga
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ctctccccgcgcgttgccgattcattaatgcagctggcacgacaggttt
cccgactggaaagcgggcagtgagcgcaacgcaattaagtttctgcagg

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