Supplementary materials

Table S1. Strains and plasmids used in this study

Name	Description	Reference
Escherichia coli		
DH5α	F- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR*17(r□ -, m□+) *phoA supE*44 *thi*-1 *gyrA*96 *relA1* λ- *tonA*	New England Biolabs (NEB)
BL21	*fhuA2 [lon] ompT gal [dcm] ΔhsdS*	NEB
HB101		NEB
HB101 pRK24	HB101 transformed with pRK24	(1)
RT1255	pMMBneo::R20291_*rsh*-His6 in DH5α	(2)
RT1270	pMMBneo::R20291_*rsh*-His6 in BL21	(2)
Clostridioides difficile		
630	Wild type	
630Δ*erm*	Wild type 630 lacking erythromycin resistance gene *ermB*	(3)
R20291	Wild type	
CEP18	pRF185::phiLOV2.12.1 in R20291	This study
CEP19	pRF185::phiLOV2.12.1 in 630Δ*erm*	This study
CEP21	PrelQ630Δ*erm*::phiLOV2.12.1 in R20291	This study
CEP22	Prsh630Δ*erm*::phiLOV2.12.1 in 630Δ*erm*	This study
CEP23	PrelQ630Δ*erm*::phiLOV2.12.1 in 630Δ*erm*	This study
CEP25	PrshR20291::phiLOV2.12.1 in R20291	This study
Plasmids		
pMMBneo	Low copy expression vector, *Ptac*, *neo* cassette, *KanR*	(2)
pRF185	*tetR*	(4)

pMMBneo::R20291_*rsh*-His6	R20291 *rsh* ligated into KpnI/PstI sites of pMMBneo	(2)
pMMBneo::R20291_*rshREL*-His6	*rel* amplified from R20291 *rsh* and ligated into KpnI/PstI sites of pMMBneo	This study
pRF185::phiLOV2.12.1	*phiLOV2.1* downstream of tetracycline-inducible promoter ligated into BamHI/KpnI sites of pRF185	(4)
PrelQ630Δ*erm*::phiLOV2.12.1	Predicted promoter upstream of 630Δ*erm* *relQ* amplified and ligated into BamHI/KpnI sites of pRF185::phiLOV2.12.1	This study
Prsh630Δ*erm*::phiLOV2.12.1	Predicted promoter upstream of 630Δ*erm* *rsh* amplified and ligated into BamHI/KpnI sites of pRF185::phiLOV2.12.1	This study
PrshR20291::phiLOV2.12.1	Predicted promoter upstream of R20291 *rsh* amplified and ligated into BamHI/KpnI sites of pRF185::phiLOV2.12.1	This study
pMSPT	Low copy expression vector	(5)
pMSPT::*as_rsh*	Antisense RNA to the mRNA of *rsh*Cd ligated into the SphI/XhoI sites of pMSPT	This study

6 Table S2. Sequences of oligonucleotide primers used in this study

Name	Sequence (5’ to 3’)	Reference
Cloning primers		
rsh_F KpnI	CAGGTACCGGTTATATGCATGATAAAG AATTACAAG	(2)
rsh_R PstI	CCCTGCAGCTA**ATGGTGATGGTGATGGTG**AT TTGTCATTCTATAAATAC	(2)
pRF185_F	CTGGACTTCATGAAAAACTAAAAAAAATATTG	This study
pRF185_R	CACCGACGAGCAAGGCAAGACCG	This study
phiLOV2.1_F	GCTAGCCCAGGTATGATTGAAAAAAGTTTTG TTATTACTG	This study
phiLOV2.1_R	CATGGATCCTTATTAAACATGATCTG	This study
Prsh_F BamHI	TATAGGATCCGGAAAGTTACCAGGTGAAGTT GA	This study
Prsh_R KpnI	ATATGGTACCCCACCTATTTTGTATAAAATTT TAATATATATG	This study
PrelQ_F BamHI	TATAGGATCCATGGCAAGCAAGTTATATCG	This study
PrelQ_R KpnI	ATATGGTACCCCTTTATTTGTTTTTTATGACCT TC	This study
relQ_F	CAAGAATTCCACTATGGAGCTTGTAATCA	This study
relQ_R	CAAGGATCCCATATTGCTCACCCTTTATTTG	This study
pMSPT_F	CTAAAGGGCAAAAGTGAGTATGG	This study
pMSPT_R	GACGAGCAAGGCAAGACC	This study
rsh_as_F	GGTATGCATGCTGCATAAAC	This study
rsh_as_R	CCTAGCTCTCGAGACCAAC	This study
rshREL_F KpnI	CAGGTACCATGAAAGAAGAAACTCAATCTG	This study
rshREL_R PstI	CCCTGCAGCTAATGGTGATGGTGATGGTGA CTATTAAATACATCTTCTTTAAGTGC	This study
qRT-PCR primers		
rpoCq_F	CTAGCTGCTCCTATGTCTCACATC	(1)
rpoCq_R	CCAGTCTCTCCTGGATCAACTA	(1)

rsh_qF	AAAATAGGTGGTTAT	This study
rsh_qR	TCAATTTTATTATCCCTCCTTTGA	This study
relQ_qF	CATTGCGGGTTCAAAGGAAAT	This study
relQ_qR	CATTGCGGGTTCAAAGGAAAT	This study

Underlined: restriction sites

Bold: sequence encoding hexa-histidine tag

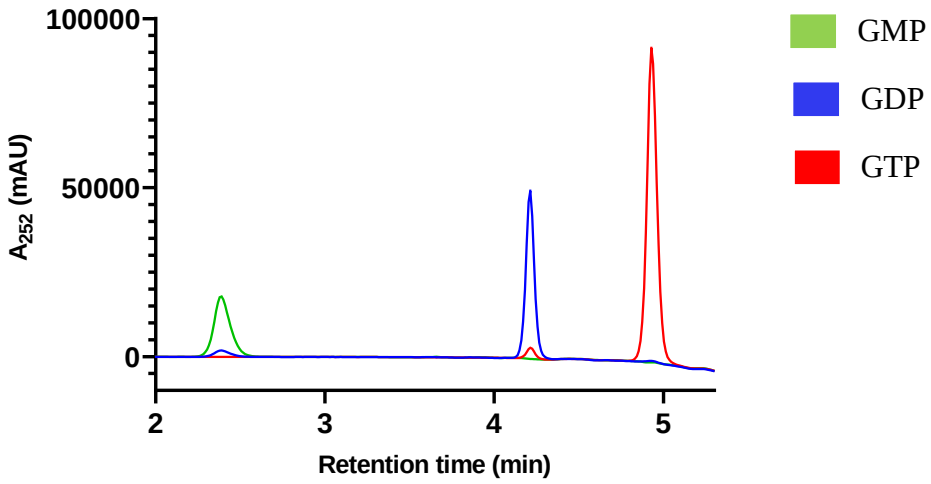


Figure S1. HPLC chromatogram of nucleotides. GTP, GDP, and GMP standards run separately at 20 µM concentration. Peaks resolved at 252 nm demonstrate that RSHCd's inability to utilize GTP for a substrate is not an artifact of substrate breakdown and/or contamination.

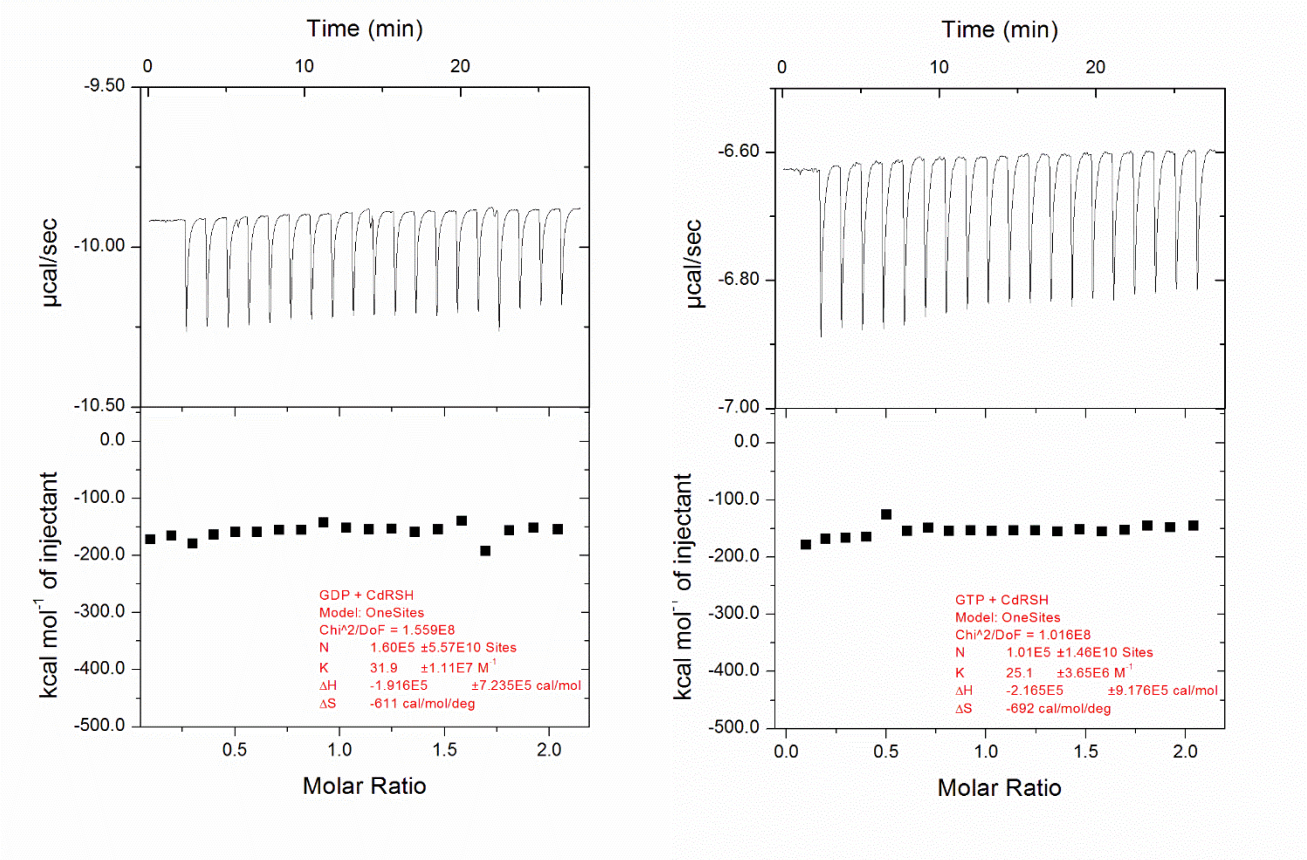
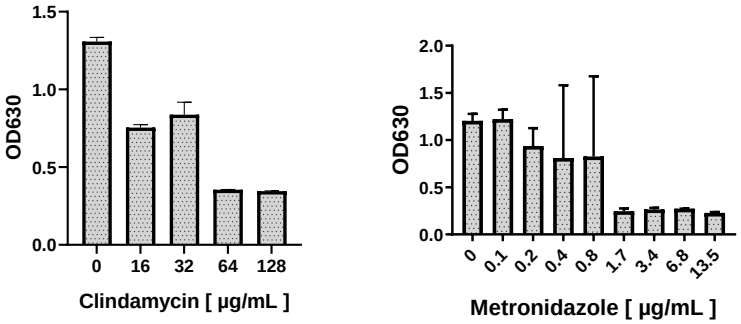


Figure S2. ITC thermogram and Wiseman plots of RSHCd interactions with GXP at 37°C. Binding of RSHCd to GDP (left) and GTP (right). The upper panels show raw data for titration of GXP with RSHCd, and the lower panels show the integrated heats of binding obtained from the raw data. The data were fitted to a single-binding site model. Each value is the average of three repeat experiments and the standard deviation ± are shown.



Sublethal concentration	Clindamycin (µg/mL)	Metronidazole (µg/mL)
0.25X	4.00	0.075
0.5X	8.00	0.15
1X	16.0	0.30

Figure S3. Sublethal concentrations for clindamycin and metronidazole against R20291. Sublethal concentrations of antibiotics for wild-type *C. difficile* strain R20291 were analyzed using the standard minimum inhibitory concentrations (MICs) determination method. ON starter culture of R20291 was inoculated (1:10) into fresh BHIS media treated with increasing concentrations of either clindamycin or metronidazole. Cells were incubated anaerobically at 37°C for 12 h to monitor growth inhibition. The sublethal concentrations are shown for four biological replicates.

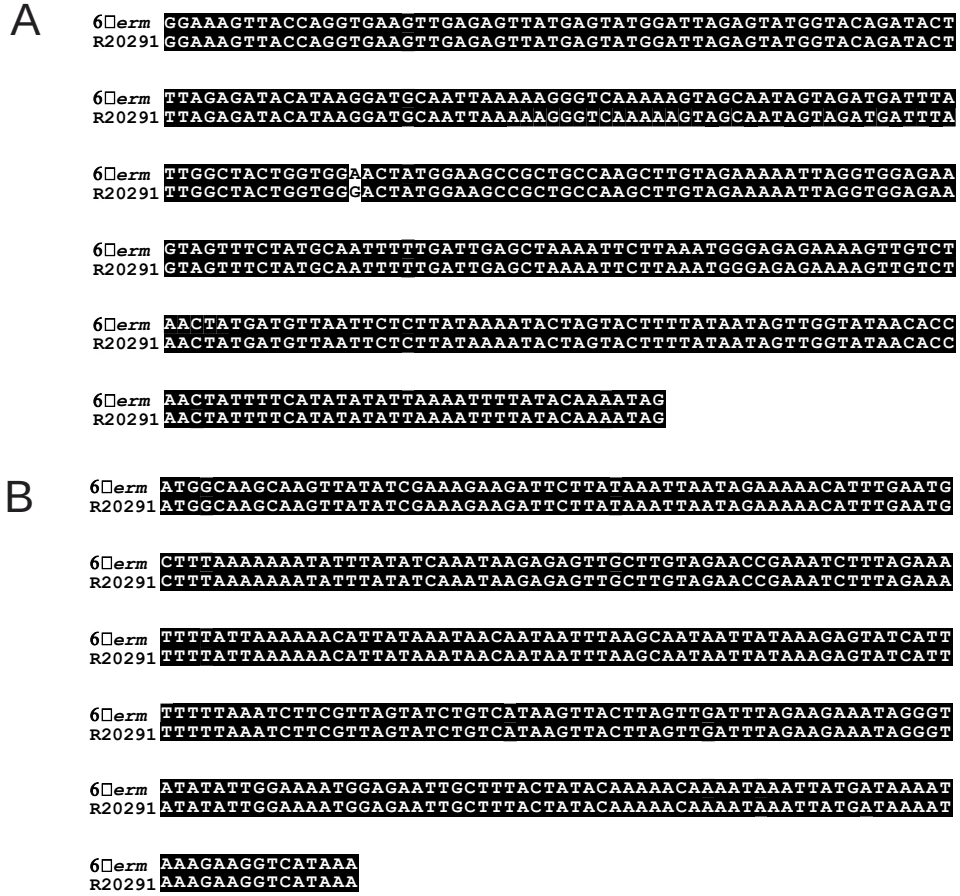


Figure S4. Sequence alignments of the upstream promoter regions used in the (A) P*rsh* and (B) P*relQ* transcriptional reporters.

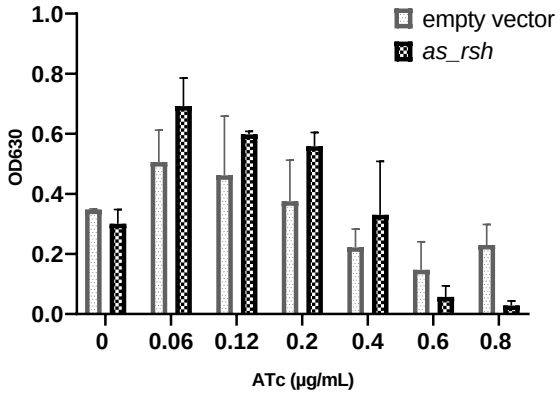


Figure S5: Minimum inhibitory concentration for ATc against *C. difficile* RNAi strains. MIC for *C. difficile* R20291 RNAi strain harboring the respective plasmids were analyzed in BHIS plus thiamphenicol (15 µg/mL) media with increasing concentrations of ATc. ON starter cultures of RNAi strains were inoculated into media (1:20) and incubated anaerobically at 37°C for 24 h to monitor growth inhibition. The MIC against empty vector (pMSPT) and asRNA vector (*as_rsh*) expressing strains are shown for two biological replicates. 0.5 µg/mL was determined to be the sublethal concentration of ATc against both RNAi strains to be used for end point growth assays.

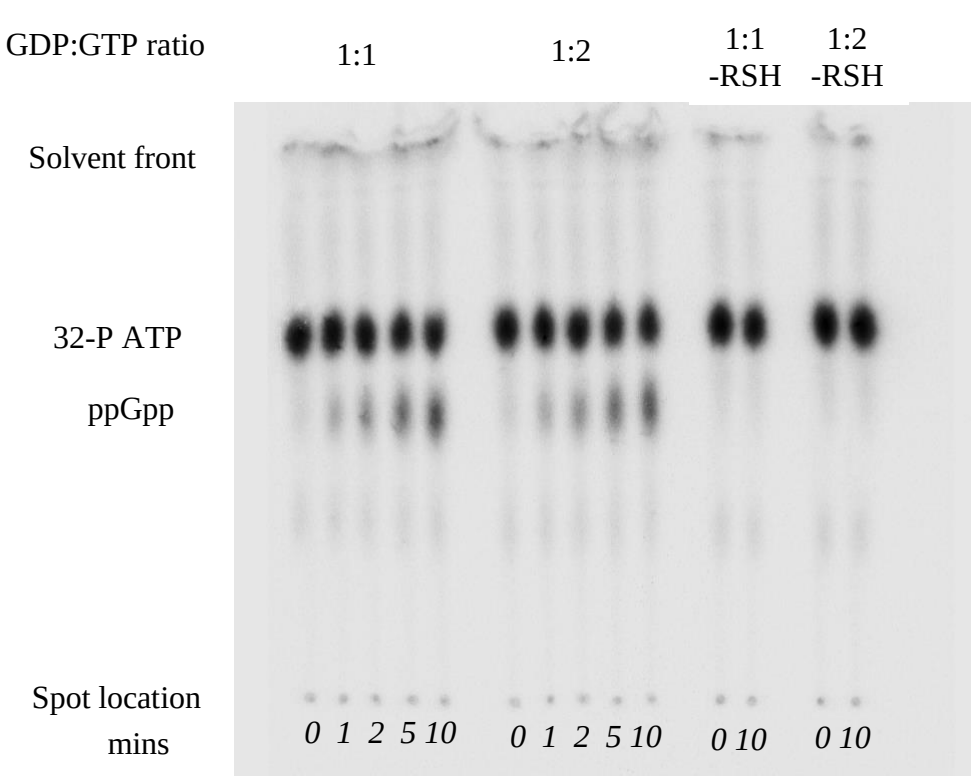


Figure S6: TLC autoradiogram of *in vitro* RSHCd transferase activity at different GDP:GTP ratio. *In vitro* transferase reaction using equimolar concentration of GDP and GTP (0.15 mM each) and 2X more GTP (0.2 mM) than GDP (0.1mM) keeping the total [substrate] equivalent to 0.3 mM. Failure to detect pppGpp suggests that RSHCd is incapable of utilizing GTP at equimolar concentration of GDP and at excess concentration of GTP than GDP.

Works cited:

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3. Hussain, H. A., Roberts, A. P., and Mullany, P. (2005) Generation of an erythromycin-sensitive derivative of Clostridium difficile strain 630 (630Deltaerm) and demonstration that the conjugative transposon Tn916DeltaE enters the genome of this strain at multiple sites. *J Med Microbiol* **54**, 137-141
4. Buckley, A. M., Jukes, C., Candlish, D., Irvine, J. J., Spencer, J., Fagan, R. P., Roe, A. J., Christie, J. M., Fairweather, N. F., and Douce, G. R. (2016) Lighting Up Clostridium Difficile: Reporting Gene Expression Using Fluorescent Lov Domains. *Scientific Reports* **6**, 23463
5. Marreddy, R. K., Wu, X., Sapkota, M., Prior, A. M., Jones, J. A., Sun, D., Hevener, K. E., and Hurdle, J. G. (2018) The fatty acid synthesis protein enoyl-ACP reductase II (FabK) is a target for narrow-spectrum antibacterials for Clostridium difficile infection. *ACS infectious diseases* **5**, 208-217