Supplemental Methods

Bacterial growth and antibiotics

Where appropriate the medium was supplemented with 15μg/ml thiamphenicol (Sigma-Aldrich, USA) or 20μg/ml of lincomycin (Sigma-Aldrich, USA). All medium was pre-incubated before use for at least 12hrs under anaerobic conditions. All *Escherichia coli* strains were grown in Luria-Bertani (LB, Bacto, USA) at 37°C supplemented with 20μg/ml chloramphenicol as required.

Anti *C. difficile* antibodies were generated against paraformaldehyde-fixed whole bacteria in mice.

Growth curves for bacteria were performed in BHIS containing 0.1M glucose. Overnight culture *C. difficile* R20291 and mutants were diluted to OD_{600} 0.01 and OD_{600} was measured every hour for 12hrs. All measurements were performed in triplicates.

Mutants and genetic complementation studies

Construction of CRG3351 and CRG1183

The *fliC*-gene and the *luxS*-gene were insertionally inactivated using the ClosTron system as described (1, 2). Plasmid pMTL007C-E2 was retargeted to yield pMTL007C-E2-fliC430s and pMTL007C-E2-luxS161a. These plasmids were conjugated into R20291 and transconjugants were selected on agar plates supplemented with thiamphenicol and cycloserine/cefoxitin. Transconjugants were then plated onto lincomycin agar plates to select for integrants. Potential integrants were verified by PCR screening and subsequent DNA sequencing using the primers *fliC*-430s-R20291-F (CCAAGCAGGAAGAAACGTTCAAGATGG) *fliC*-430s-R20291-R (GCTGCATCTGTTCCAG AAGTTCC), *luxS*_95_F (AGTAACTAAATTTGACTTGAGATTTTTACAGCC) and *luxS*_262_R (AACCAATCTTAACTGTTTTGGCATCTACATCTCCCC).

Construction of plasmid vector pMTL960

The *catP* gene from pMTL5402F (1) was cloned into the pCRII-TOPO vector (Invitrogen) and confirmed by sequencing. The origin of transfer (*oriT*) and *traJ* sequence required for conjugal transfer was subsequently cloned in as an *Eco*RI fragment from pMTL9301 (3). The *catP-oriT-traJ* fragment was excised from the pCRII-TOPO vector as an *Apa*LI/*Eco*RV fragment and ligated together with an *Apa*LI/*Sma*I fragment from pMTL9301 which contained the *C. difficile* pCD6 replicon and the gram-negative replicon *Col*EI. The resulting vector was named pMTL960.

Complementation of mutants

For complementation studies for CRG1166, plasmid pMTL-DB1 (4) was introduced into the mutant, resulting in strain CRG1634. Chromosomal complementation of CdiR20291Δ*cwp84* with *cwp84* was performed as described (5) to yield CdiR20291Δ*cwp84*-C. A 724-bp fragment surrounding the putative *luxS* structural gene (456-bp) and 5-noncoding region (268-bp) was amplified by PCR using oligonucleotide primer F1_*luxS* (TAAAGAATGCGGCCGCGTACGATTATGTGATATAAATA TTATAAC) and R1_*luxS* (TAAAGAATCTCGAGTTATTCTCCATATATATTTAAAGAAAATCC) and cloned using *Not*I and *Xho*I into pMTL-84151, resulting in plasmid pMTL-TD1 (6). This plasmid was conjugated into the *luxS* mutant CRG1183 to yield CRG1183-C. Fragments containing the native promoter and full length genes for *fliC* and *spo0A* were cloned into plasmids pMTL-84151 and pMTL-960, respectively. The *spo0A* sequence was amplified by PCR using genomic DNA from *C. difficile* 630Δ*erm* as template and primers Cdi-*spo0A*-F1 (AACTAGTGGTATTTTTATAGATGAAATG ATAAAATTGTAGGTGAGG) and Cdi-*spo0A*-R1 (GGATCCTCAGTTTACAACTTGTAAAGACA CATACTATATCC). The resulting product comprised the *spo0A* open reading frame with 174-bp of upstream sequence and 205-bp of downstream sequence. This was cloned into the pCR2.1-TOPO vector (Invitrogen) and confirmed by sequencing. The *spo0A* complementation construct was subsequently excised as a *Spe*I/*Bam*HI fragment and cloned into similarly digested pMTL960 and conjugate into *spo0A* mutant CRG1375 to yield CRG1375-C. For complementation of the *fliC* mutant,

a 973-bp fragment encompassing the *fliC* open reading frame (873bp) with the 100-bp 5' noncoding region most likely containing the *fliC* promoter, was amplified with primers *Not*I-p*fliC*-630-F (ACTGCGGCCGCAGTTATAGATTAACTTGTCCG) and *Xho*I-*fliC*-630-R (GAATAAAAAAGA AAGGATAAGGCTTTGCCTCGAGGACAG) and cloned using *Not*I and *Xho*I to generate pMTL-SB1. After conjugation into CRG3351, this yielded strain CRG3359.

All plasmids were transferred into *C. difficile* by conjugation as described previously (3).

References

1. **Heap, J. T., O. J. Pennington, S. T. Cartman, G. P. Carter, and N. P. Minton.** 2007. The ClosTron: a universal gene knock-out system for the genus Clostridium. J Microbiol Methods **70:**452-64.
2. **Heap, J. T., S. A. Kuehne, M. Ehsaan, S. T. Cartman, C. M. Cooksley, J. C. Scott, and N. P. Minton.** 2010. The ClosTron: Mutagenesis in Clostridium refined and streamlined. J Microbiol Methods **80:**49-55.
3. **Purdy, D., T. A. O'Keeffe, M. Elmore, M. Herbert, A. McLeod, M. Bokori-Brown, A. Ostrowski, and N. P. Minton.** 2002. Conjugative transfer of clostridial shuttle vectors from Escherichia coli to Clostridium difficile through circumvention of the restriction barrier. Mol Microbiol **46:**439-52.
4. **Burns, D. A., J. T. Heap, and N. P. Minton.** 2010. SleC is essential for germination of Clostridium difficile spores in nutrient-rich medium supplemented with the bile salt taurocholate. J Bacteriol **192:**657-64.
5. **Yen Kuan Ng, M. Ehsaan S. Philip, C. Janoir, J. T. Heap, A. Collignon, S. Cartman, N. P. Minton*** 2012. Expanding the Repertoire of Gene Tools for Precise Manipulation of the

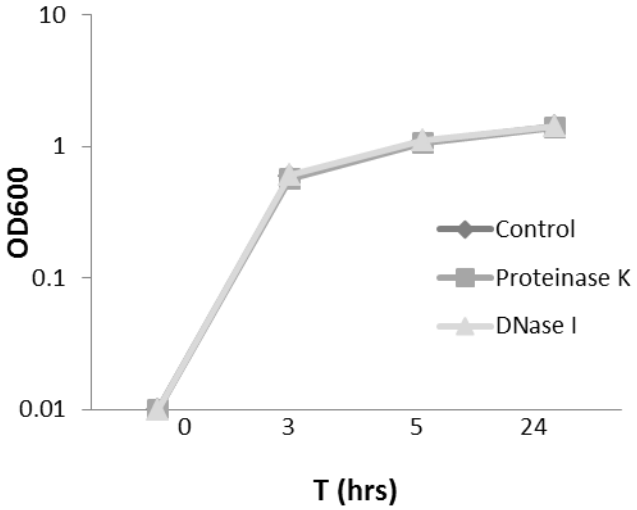
Clostridium difficile Genome: Allelic Exchange Using PyrE Alleles. Manuscript under submission

6. **Heap, J. T., O. J. Pennington, S. T. Cartman, and N. P. Minton.** 2009. A modular system for Clostridium shuttle plasmids. J Microbiol Methods **78:**79-85.

Table 1. Bacterial strains and plasmids.

Strain or plasmid	**Relevant properties**	**Source or reference (Supplementary)**
Strains		
E. coli CA434	Conjugation donor	(6)
CdiR20291Δ*cwp84* (Δ*cwp84*)	*C. difficile* R20291 strain with an in frame deletion of *cwp84* from 642-bp to 1887-bp	(4)
CRG3351(*fliC*)	CdiR20291-*fliC*430s::CT	This study
CRG1183 (*luxS*)	CdiR20291-*luxS*161a::CT	This study
CRG1166 (*sleC*)	CdiR20291-*sle*C128a::CT	(1)
CRG1375 (*spo0A*)	CdiR20291-*spo0A*178a	(3)
CdiR20291Δ*cwp84*-C (Δ*cwp84*-C)	*C. difficile* R20291 Δ*cwp84* complemented with *cwp84* on the chromosome	(2)
CRG1183-C (*luxS*-C)	CRG1183 containing pMTL-TD1	This study
CRG1634 (*sleC*-C)	CRG1166 containing pMTL-DB1	(1)
CRG3359 (*fliC*-C)	CRG3351 mutant containing pMTLSB1	This study
CRG1375-C (*spo0A*-C)	CRG1375 containing pMTL960:*spo0A*(Cdi)	This study

Plasmids		
pMTL84151	Clostridium modular plasmid containing *catP*	(5)
pMTL960	Clostridium modular plasmid containing *catP*	This study
pMTL-DB1	pMTL84151 containing 1,272-bp *sleC* coding region and 244-bp upstream promoter region	(1)
pMTL-TD1	pMTL84151 containing 456-bp *luxS* coding region and 124-bp upstream promoter region	This study
pMTLSB1	pMTL84151 containing 873-bp *fliC* coding region and 100-bp upstream *fliC* promotor region	This study
pMTL960:spo0A(Cdi)	pMTL-960 containing *spo0A* open reading frame with 174-bp of upstream sequence and 205-bp of downstream sequence	This study



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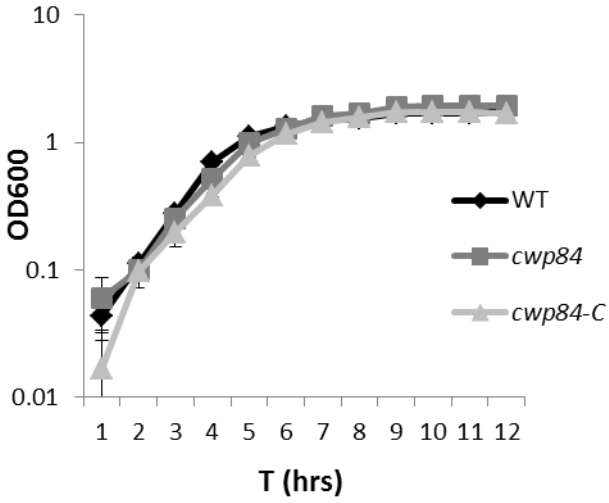
94 **Figure S1.** Growth curves of R20291 in BHI in presence of Proteinase K (0.1mg/ml) or DNase I

95 (2U/ml).



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97 **Figure S2.** Growth curves for Δ*cwp84* mutant and complemented (*Δcwp*-C) strains.

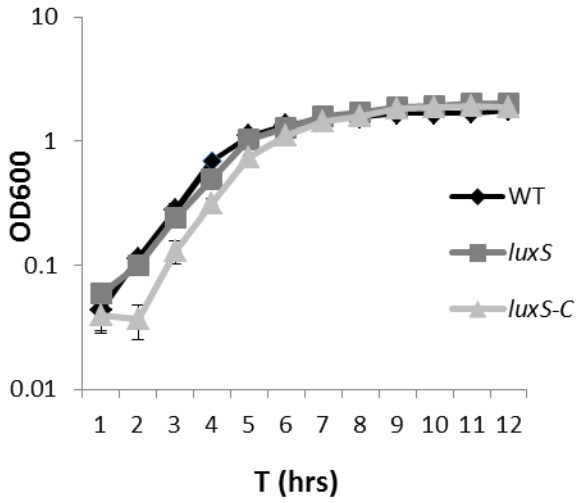


Figure S3. Growth curves for *luxS* mutants and complemented *(luxS*-C) strains.

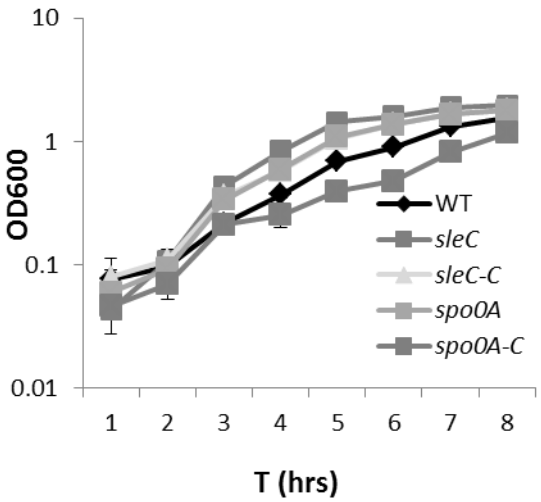


Figure S4. Growth curves for sporulation and germination mutants, *spo0A* and *sleC* and complemented strains (spo0A-C and *sleC*-C).

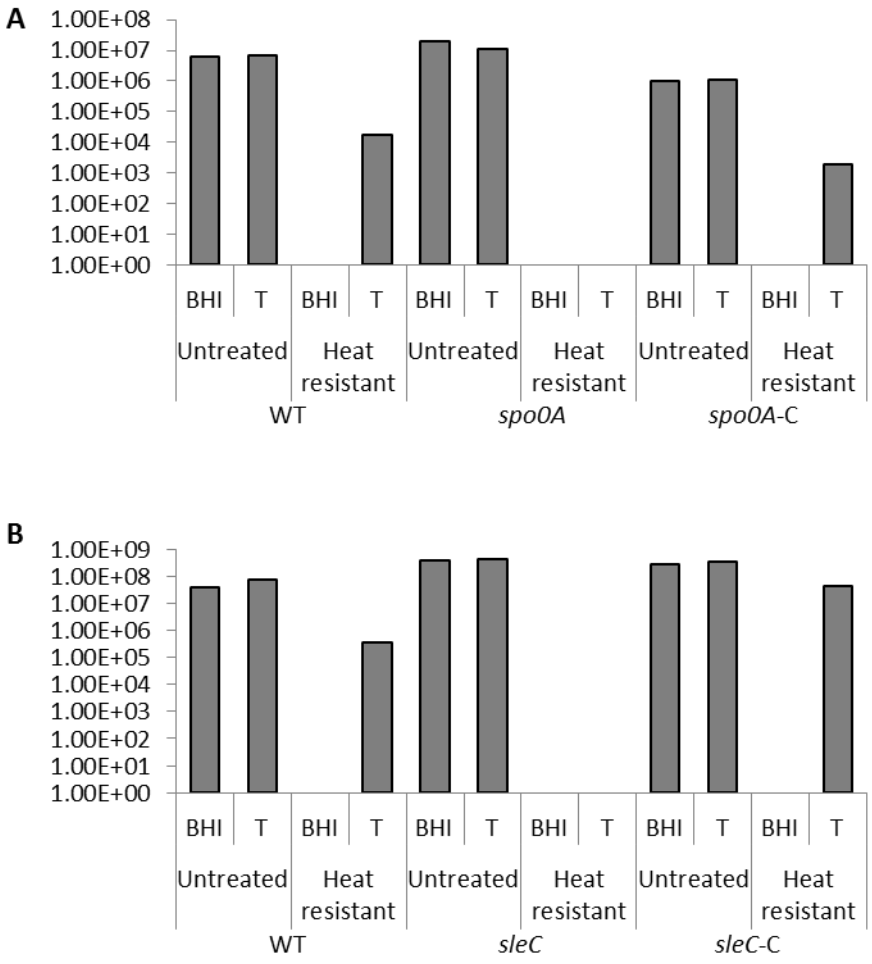
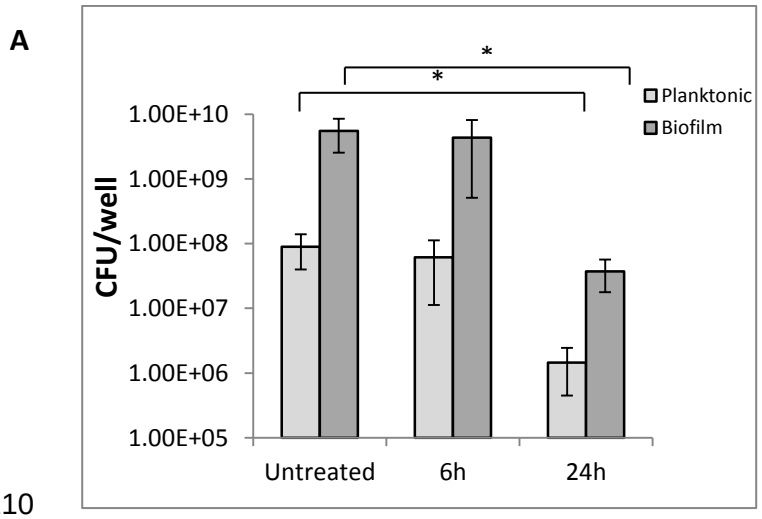


Figure S5. Germination assays for *spo0A* and *sleC* mutants. Numbers of colony forming units CFU/ml obtained from sporulating cultures, with and without heat treatment of *C. difficile* R20291, *spo0A* and *spo0A* complemented strain (*spo0A*-C) (A), and *sleC*, *sleC* complemented strain (*sleC*-C) (B) after 48hrs of incubation in on BHI in presence or absence of sodium taurocholate (T).



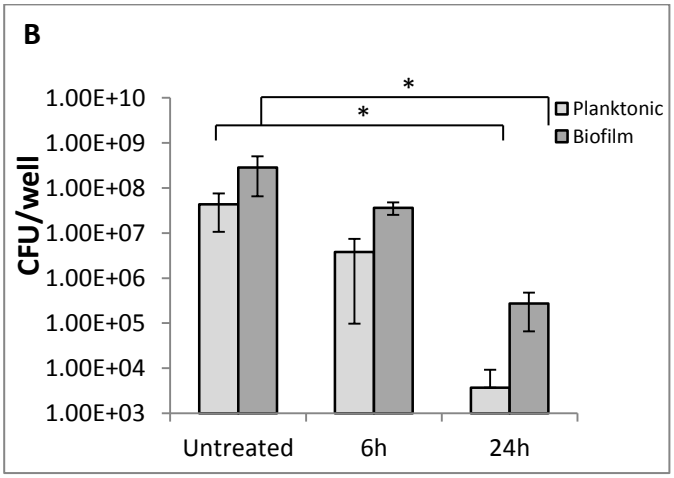


Figure S6. Antibiotic resistance of *C. difficile* biofilms. (A) *Clostridium difficile* 1-day-old (B) to 3-day-old (C) biofilms and the corresponding planktonic growth were exposed to 20µg/ml vancomycin (100 times the MIC) for 6hrs and 24hrs. CFU counts before (untreated) and after (6h and 24h) treatment with vancomycin are shown. Results are presented in log scale and are representive of results from 3 independent experiments, performed in triplicates.