Supplemental Information

Figure S1: Growth of *C. difficile* strains in TY medium supplemented with glucose.

C. difficile R20291 (wild-type) (●), *C. difficile* KNM6 (Δ*selD*) (■), and *C. difficile* KNM9 (Δ*selD*::*selD*$^{+}$) (▲) were grown in TY medium supplemented with glucose and growth was monitored over a 24 hour period. Data points represent the average from three independent experiments and error bars represent the standard deviation from the mean.

Figure S2: Deletion of *spo0A* in *C. difficile* R20291.

DNA was isolated from *C. difficile* R20291 (wild-type) and *C. difficile* KNM10 (Δ*spo0A*). The region surrounding the *spo0A* gene was amplified from the chromosome, and the resulting DNA was separated on an agarose gel. A clean deletion of *spo0A* is indicated by a faster-migrating DNA band while wild-type is indicated by a slower-migrating DNA band.

Table S1: Complete list of gene expression fold-change from RNA-seq of wild-type and *selD* mutant strains.

Shown in additional Microsoft Excel file.

Table S2: Strains and plasmids used in this study.

Strain	Description/Phenotype	Source or Reference

E. coli DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	(1)
E. coli HB101 pRK24	*lavYI galK2 xyl-6 mtl-I repsL20* carrying pRK24	(2)
B. subtilis BS49	Tn*916* donor strain, Tet^R	(3)
C. difficile R20291	Wild type, ribotype 027	(4)
C. difficile KNM6	*selD* targeted CRISPR-*cas9* mutant	(5)
C. difficile KNM9	KNM6 strain CRISPR-*cas9* restoration of *selD* at its native locus, Δ*selD*::*selD*$^+$	This study
C. difficile KNM10	*spo0A* targeted CRISPR-*cas9* mutant	This study
Plasmids		
pIA33	P_{xyl}::*dCas9-opt* P_{gdh}::*sgRNA-rfp catP*	(6)
pJK02	P_{tet}-*Cas9-opt* P_{gdh}-*sgRNA colE1 pCD6 traJ catP*	(5)
pJS116	*B. subtilis-C. difficile* shuttle vector (pCD6 ColE1 *Tn916 oriT* Cm^R)	(7)
pKM126	*tn916* oriT in pJK02	(5), This study
pKM142	*selD* with 500 bp upstream in pJS116	(5)
pKM181	*selD* complementing homology region in pKM126	This study
pKM183	sgRNA for targeting region surrounding *selD* deletion in pKM181	This study
pKM194	xylose-inducible P_{xyl} promoter in pKM183	This study
pKM197	xylose-inducible P_{xyl} promoter in pKM126	(8), This study
pKM213	*spo0A*-targeting sgRNA in pKM197	This study
pKM215	*spo0A* deletion homology region in pKM213	This study

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21 **Table S3: Oligonucleotides used in this study.**

Oligonucleotide	Sequence	Reference
5'Tn916ori	AAGCGGAAGAGCGCCCAATACGCAGGGCCCTA ACATCTTCTATTTTTCCCA	(5)
3'Tn916ori	TATCTACAATTTTTTTATCCTGCAGGGGGCCCCT AAAGGGAATGTAGATAAATTATTAG	(5)
5'selD_comp	CAATTTTTTTATCAGGAAACAGCTATGACCGCG GCCGCACCTAAAATAGGTGAAGCAAC	This study
3'selD_comp 2	GGTCTTAAGCGATCGCGCATGTCTGCAGGCCT CGAGCGCTGCATTATTATTTACAA	This study

CRISPR_selD_comp2	GTGTGCTATAATTAAACTGTAAAACGCGTAGCCGCTAAAATAGGGCCAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTATGGAGAAATCTAGATCAGCATGATGTCTGACTAGACGCGTAAGCTCTGCAACTATTTTTAGAT	This study
5'selDcomp_HR_xylR 2	GTACTAGTTGTAAATAATAATGCAGCGCTCGAGCTAGCATAAAAATAAGAAGCCT	This study
3'cas9_Pxyl 2	TAATCCTATACTATATTTTTTATCCATTTAATTAACTCTCCTCTTTACCCTCCTT	This study
5'pyrE_HR_xylR 2	CATTCAAAAGAAGGAAGAACATCAATGCTTCTCGAGCTAGCATAAAAATAAGAAGCCT	This study
CRISPR_spo0A_2	GTGTGCTATAATTAAACTGTAAAACGCGTGACATGCAATAGAGGTTGCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTATGGAGAAATCTAGATCAGCATGATGTCTGACTAGACGCGTAAGCTCTGCAACTATTTTTAGAT	This study
5' spo0A_UP	TTTTTTTATCAGGAAACAGCTATGACCGCGGCCGCCAGAAAACCATAATAAAGAGTTTAA	This study
3' spo0A_UP	TGTCTTGTCCTGTTGAATGTCTTCCTTCTGCTAAAAAACATCTTCTTATTACAGAAAACT	This study
5' spo0A_DN	GATGTTTTTTAGCAGAAGGAAGACATTCAACAGGACAAGACATAAAAAGTAAGGC	This study
3' spo0A_DN	AATGCAGGCTTCTTATTTTTATGCTAGCTCGAGGATTTATAACTGCTATTTCCCC	This study
5’selD	GAGCTTCCTAAAAATGAAGTAAATATCAATAAACAG	(5)
3’selD	TTTTGCTCAAAACAATCACTCTTTCTCTATAATATT	(5)
5'spo0A_del	CAAATAATTCAGAGCTAGGTATAAGTGGTAATAT	This study
3'spo0A_del	CAATGCCTTAATTAAAAAGCCTTACTTTTTATGTCTTG	This study
5' tcdB	TTACATTTTGTTTGGATTGGAGGTC	(5)
3' tcdB	AGCAGCTAAATTCCACCTTTCTACC	(5)
5' catP 3	ATGGTATTTGAAAAAATTGATAAAAATAG	(5)
3' catP 2	TTAACTATTTATCAATTCCTGCAATTCG	(5)
5'tetR_CO_cas9	CTGAGCTCAATAATACTAGGAGGTTTTTTTAATTAAATGGATAAAAAATATAGTATAGGATTAGATATAGGAAC	(5)
3'COcas9 (975)	GTAATGTTAAATCTTGATGATGTTCATC	This study
5'gdh	TGCAGGCTTCTTATTTTTATGGTTTAAACGGTTTTAGCTGGGATATCG	(5)
3'gRNA 2	CATCTAAAAATAGTTGCAGAGCTTACGCGTCTAGTCAGACATCATGCTGA	(5)

5'rpoB_qPCR	GAGTGTAAAGAGAGAGATGC	(9)
3'rpoB_qPCR	CTTCCGCATAGTAAACACC	(9)
5' tcdB_qPCR	GGCAAATGTAAGATTTCGTGTTCA	(10)
3' tcdB_qPCR	TCGACTACAGTATTCTCTGAC	(10)
5'0963_qPCR	CAGACTGTTGCAGATAGCATTGAGTA	This study
3'0963_qPCR	CAACAACAAATCTGTTTACACCTTGA	This study
5'0962_qPCR	TCAGGCTCCTACAACACTTTTATTTG	This study
3'0962_qPCR	TCTGCATTACTTTCCTCGATTATCTC	This study
5'prdB_qPCR	GGAAGAGGGAGTAGACGGTGTAGTT	This study
3'prdB_qPCR	ACGATCACGGCAGTTCTATGG	This study
5'grdB_qPCR	TATAGCAGGAGTTATGGATTTAACAGAAGAG	This study
3'grdB_qPCR	CTAAATTTGCATACACTGGGTCATATC	This study
5'grdA_qPCR	TTTCGCTGGACCACTTGCT	This study
3'grdA_qPCR	TGGTTCCTCAACAACGTGGTAA	This study
5'mtlF_qPCR	CATATATGGGAATGGGAGTTGCTAT	This study
3'mtlF_qPCR	TTTCTCCATCAAAATCTATACCATTAGG	This study

1. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166:**557-580.
2. **Bouillaut L, McBride SM, Sorg JA.** 2011. Genetic manipulation of *Clostridium difficile*. Curr Protoc Microbiol **Chapter 9:**Unit 9A 2.
3. **Haraldsen JD, Sonenshein AL.** 2003. Efficient sporulation in *Clostridium difficile* requires disruption of the *sigmaK* gene. Molecular Microbiology **48:**811-821.
4. **Stabler RA, He M, Dawson L, Martin M, Valiente E, Corton C, Lawley TD, Sebaihia M, Quail MA, Rose G, Gerding DN, Gibert M, Popoff MR, Parkhill J, Dougan G, Wren BW.** 2009. Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. Genome Biol **10:**R102.
5. **McAllister KN, Bouillaut L, Kahn JN, Self WT, Sorg JA.** 2017. Using CRISPR-Cas9-mediated genome editing to generate *C. difficile* mutants defective in selenoproteins synthesis. Scientific Reports **7:**14672.
6. **Muh U, Pannullo AG, Weiss DS, Ellermeier CD.** 2019. A xylose-inducible expression system and a CRISPRi-plasmid for targeted knock-down of gene expression in *Clostridioides difficile*. J Bacteriol.
7. **Francis MB, Allen CA, Shrestha R, Sorg JA.** 2013. Bile acid recognition by the *Clostridium difficile* germinant receptor, CspC, is important for establishing infection. PLoS Pathog **9:**e1003356.
8. **Bhattacharjee D, Sorg JA.** 2020. Factors and conditions that impact electroporation of *Clostridioides difficile* strains. mSphere **5**.
9. **Fimlaid KA, Bond JP, Schutz KC, Putnam EE, Leung JM, Lawley TD, Shen A.** 2013. Global analysis of the sporulation pathway of *Clostridium difficile*. PLoS Genet **9:**e1003660.
10. **Edwards AN, Tamayo R, McBride SM.** 2016. A novel regulator controls *Clostridium difficile* sporulation, motility and toxin production. Mol Microbiol **100:**954-971.

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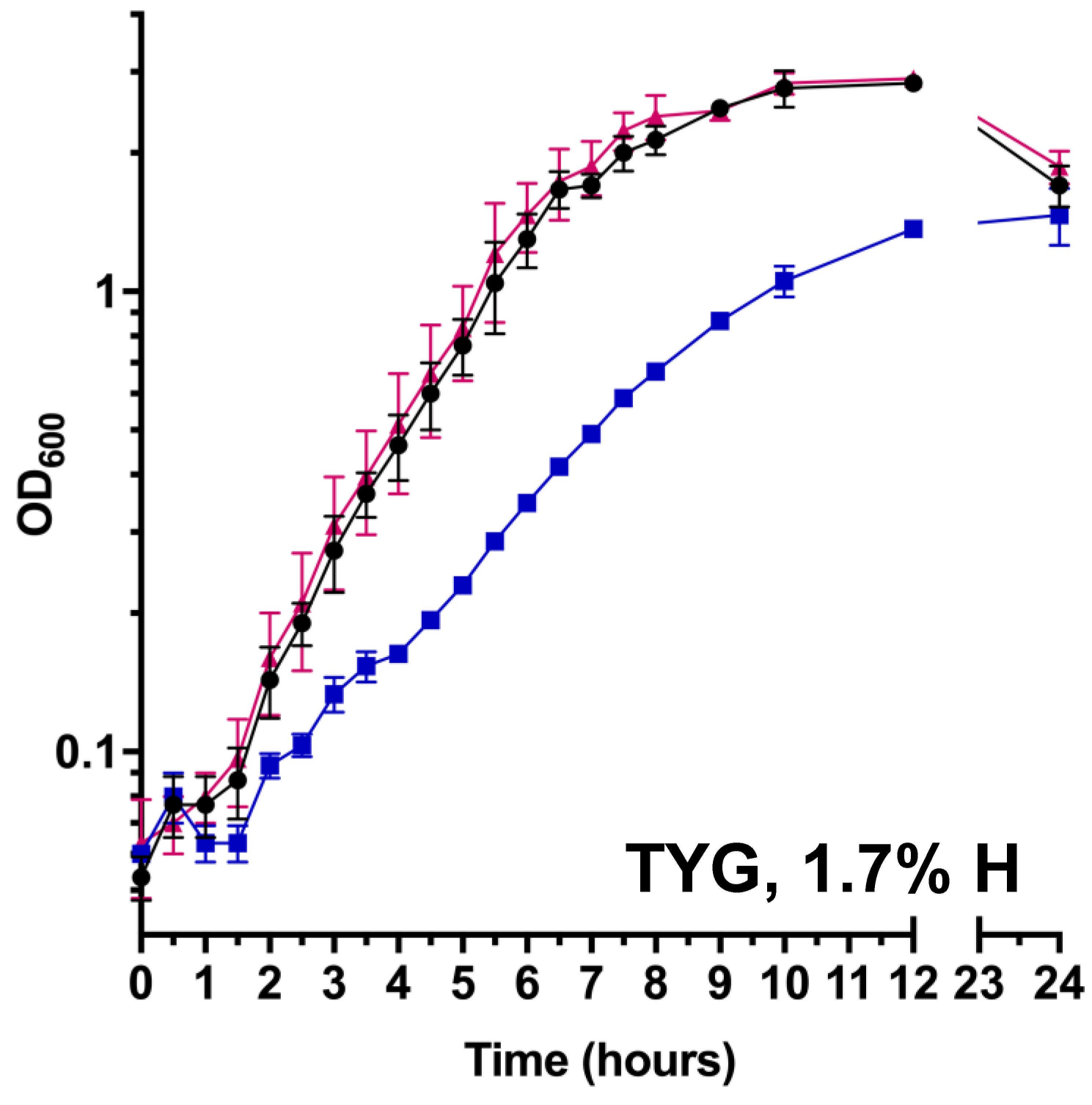
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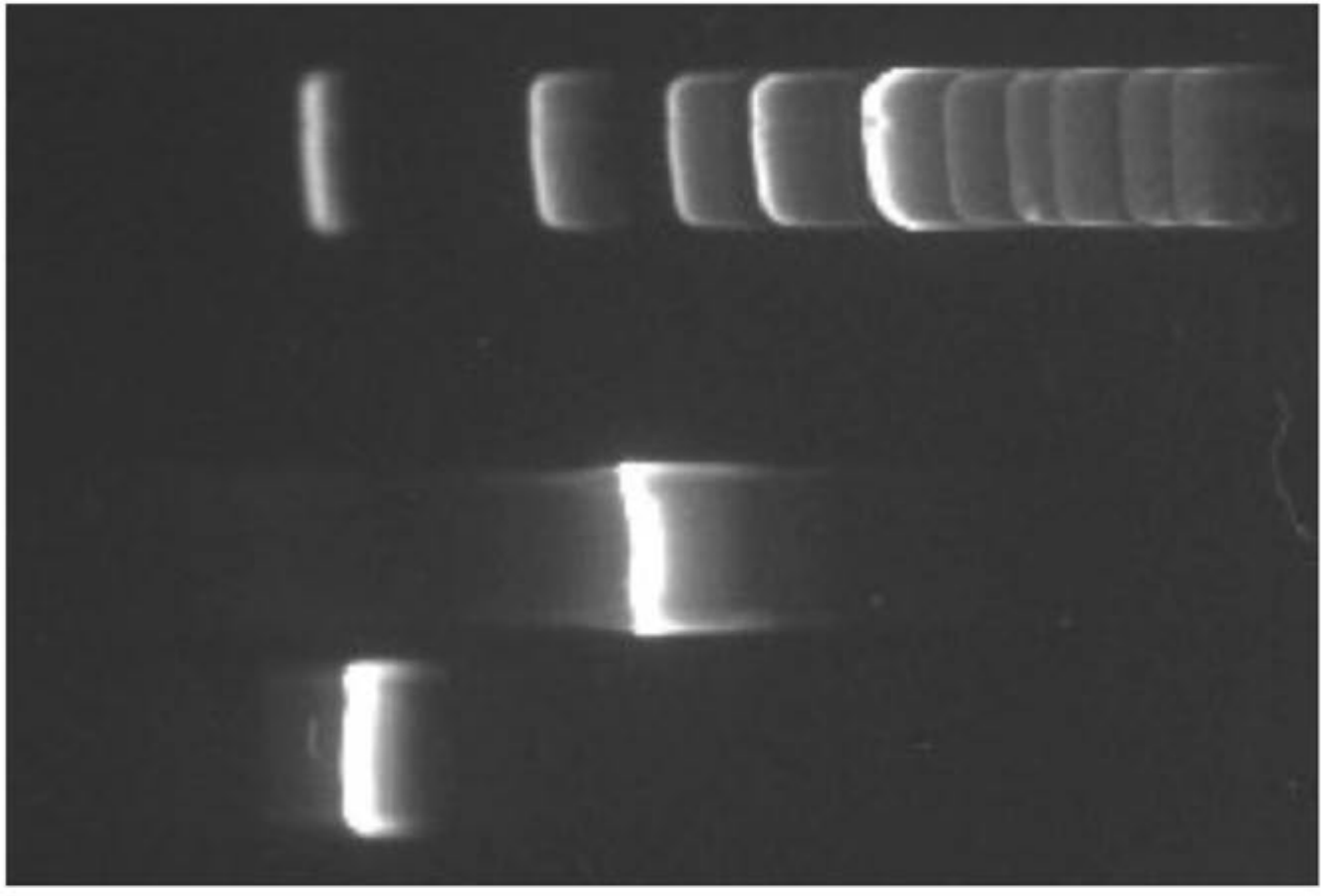
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