Supplementary File S1: Vector Construction

pMC421: A 600 bp fragment containing the upstream region of the *sinR* (CD2214) operon was amplified from *C. difficile* 630Δ*erm* genomic DNA using primers oMC1008 and oMC1009. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC426: A 400 bp fragment containing the upstream region of the *oppB* (CD0853) operon was amplified from *C. difficile* 630Δ*erm* genomic DNA using primers oMC1012 and oMC1015. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC451: A 600 bp fragment containing the upstream region of the *appA* (CD2672) operon was amplified from *C. difficile* 630Δ*erm* genomic DNA using primers oMC1025 and oMC1026. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC453: A 600 bp fragment containing the upstream region of the *appA* operon was amplified from *C. difficile* UK1 genomic DNA using primers oMC1025 and oMC1026. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC463: A 400 bp fragment containing the upstream region of the *oppB* operon was amplified from *C. difficile* UK1 genomic DNA using primers oMC1012 and oMC1015. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC474: A 250 bp fragment containing the upstream region of the *oppB* (CD0853) operon was amplified from *C. difficile* 630Δ*erm* genomic DNA using primers oMC1012 and oMC1074. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

MC476: A 170 bp fragment containing the upstream region of the *oppB* (CD0853) operon was amplified from *C. difficile* 630Δ*erm* genomic DNA using primers oMC1012 and oMC1076. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC477: A 150 bp fragment containing the upstream region of the *oppB* (CD0853) operon was amplified from *C. difficile* 630Δ*erm* genomic DNA using primers oMC1012 and oMC1077. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC478: A 250 bp fragment containing the upstream region of the *oppB* (CD0853) operon was amplified from *C. difficile* UK1 genomic DNA using primers oMC1012 and oMC1074. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC480: A 170 bp fragment containing the upstream region of the *oppB* (CD0853) operon was amplified from *C. difficile* UK1 genomic DNA using primers oMC1012 and oMC1076. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC481: A 150 bp fragment containing the upstream region of the *oppB* (CD0853) operon was amplified from *C. difficile* UK1 genomic DNA using primers oMC1012 and oMC1077. This region was cloned into pMC358 using *Bam*HI and *Eco*RI.

pMC535: A 600 bp fragment containing the upstream region of the *sinR* (CD2214) operon was generated through splicing and overlap extension PCR from *C. difficile* 630Δ*erm* genomic DNA to generate a C to A transversion 290 bp upstream the *sinR* translational start site using primers oMC1008, oMC1009, oMC1178, and oMC1179. This region was cloned into pMC358 using *Bam*HI and *Eco*RI

pMC536: A 600 bp fragment containing the upstream region of the *sinR* (CD2214) operon was generated through splicing and overlap extension PCR from *C. difficile* 630Δ*erm* genomic DNA to generate a C to A transversion 290 bp upstream the *sinR* translational start site using primers oMC1008, oMC1009, oMC1178, and oMC1179. This region was cloned into pMC358 using *Bam*HI and *Eco*RI

pBL18: pSMB47 was digested with *Eco*RI and *Nco*I. The ends were blunted and the plasmid was self-ligated generating pBL18.

pBL26: The *catP* gene of pMMOrf-Cat was amplified using 5'catP2 and ITR primers. This region was cloned into pBL18 using *Sph*I.

pMMOrf-Cat: 950 bp region containing *catP* and its promoter was amplified from pJIR1456 using 5'catP2 and 3'catP. This region was cloned into the *Sma*I site of pMMOrf.

pND3: A 983 bp fragment containing the promoter and coding sequencing of *codY* was amplified from *C. difficile* JIR8094 genomic DNA using primers oLB275 and oLB276. This region was cloned into pBL26 using *Bam*HI and *Hind*III.

Figure. S2

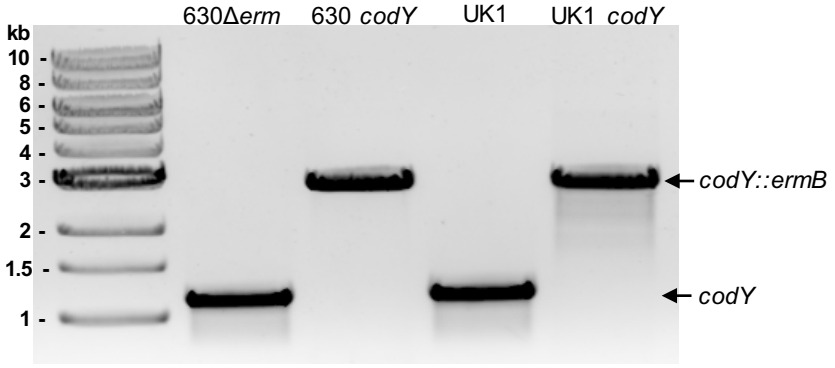

Figure S2. Confirmation of *codY* disruption. PCR amplification of *codY* from 630Δ*erm,* MC364 (630Δ*erm codY::ermB*), UK1, and LB-CD16 (UK1 *codY::ermB*) genomic DNA using primers oMC425/426, demonstrating insertion of the Targetron-based intron in mutants.

Figure. S3

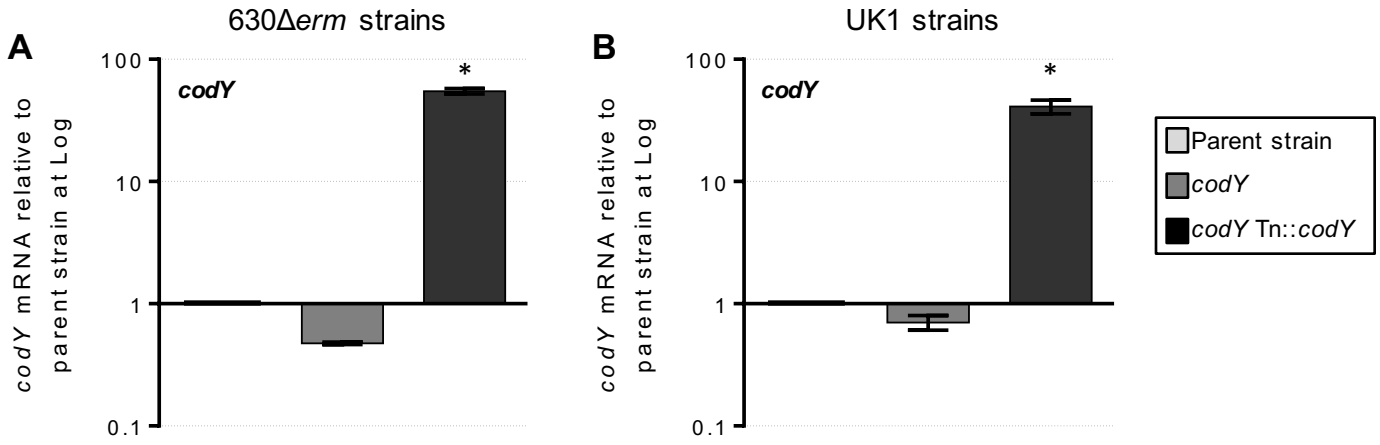

Figure S3. Analysis of *codY* expression. qRT-PCR expression analysis of *codY* for (**A**) 630Δ*erm,* MC364 (630Δ*erm codY::ermB*) and MC442 (630Δ*erm codY* Tn::*codY*) and **B**) UK1, LB-CD16 (UK1 *codY::ermB*), and MC443 (UK1 *codY* Tn::*codY*). Strains were grown in 70:30 liquid sporulation medium and samples for RNA isolation were taken during logarithmic growth (OD_{600} ~0.5). The means and standard error of the means of four biological replicates are shown. A one-way ANOVA followed by Dunnett's multiple comparison test was used to compare the parent strain with the *codY* mutant or the parent strain with the respective complemented strain. *, $P \leq 0.05$.

Figure. S4

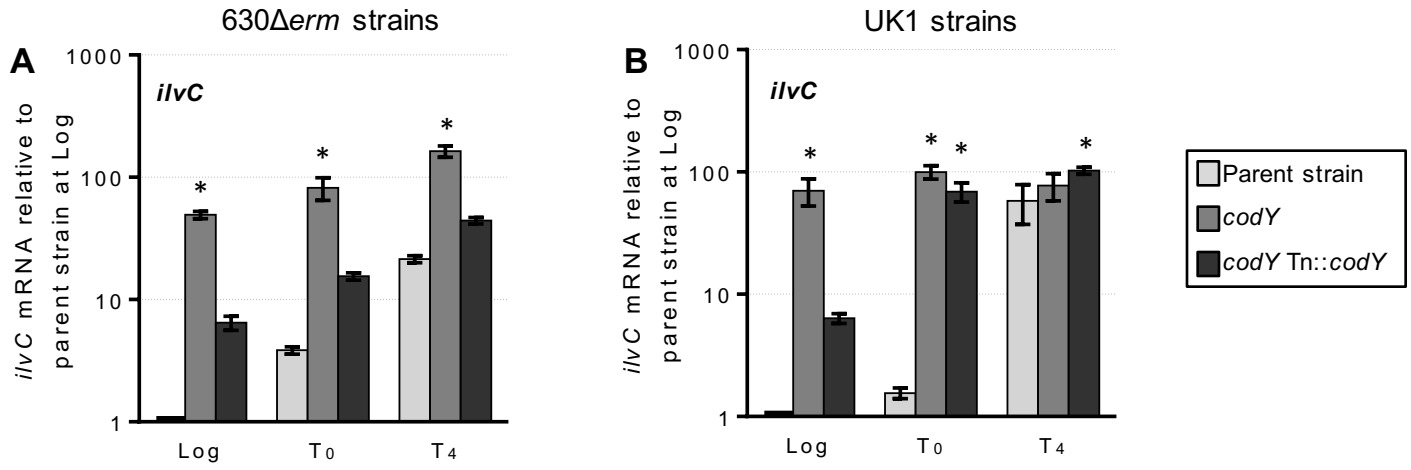

Figure S4. Loss of CodY-dependent regulation of *ilvC*. qRT-PCR expression analysis of *ilvC* in **A**) 630Δ*erm,* MC364 (630Δ*erm codY::ermB*), MC442 (630Δ*erm codY* Tn::*codY*) and (**B**) UK1, LB-CD16 (UK1 *codY::ermB*) and MC443 (UK1 *codY* Tn::*codY*) grown in 70:30 liquid sporulation media. Samples for RNA isolation were taken during logarithmic growth (OD_{600} ~0.5), transition to stationary phase (T_0), and four hours after the transition into stationary phase (T_4). The means and standard error of the means of four biological replicates are shown. A two-way repeated measures ANOVA, followed by Dunnett's multiple comparison test was used to compare parent strain with the *codY* mutant or the parent strain with the respective complemented strain. *, $P \leq 0.05$.

Figure. S5

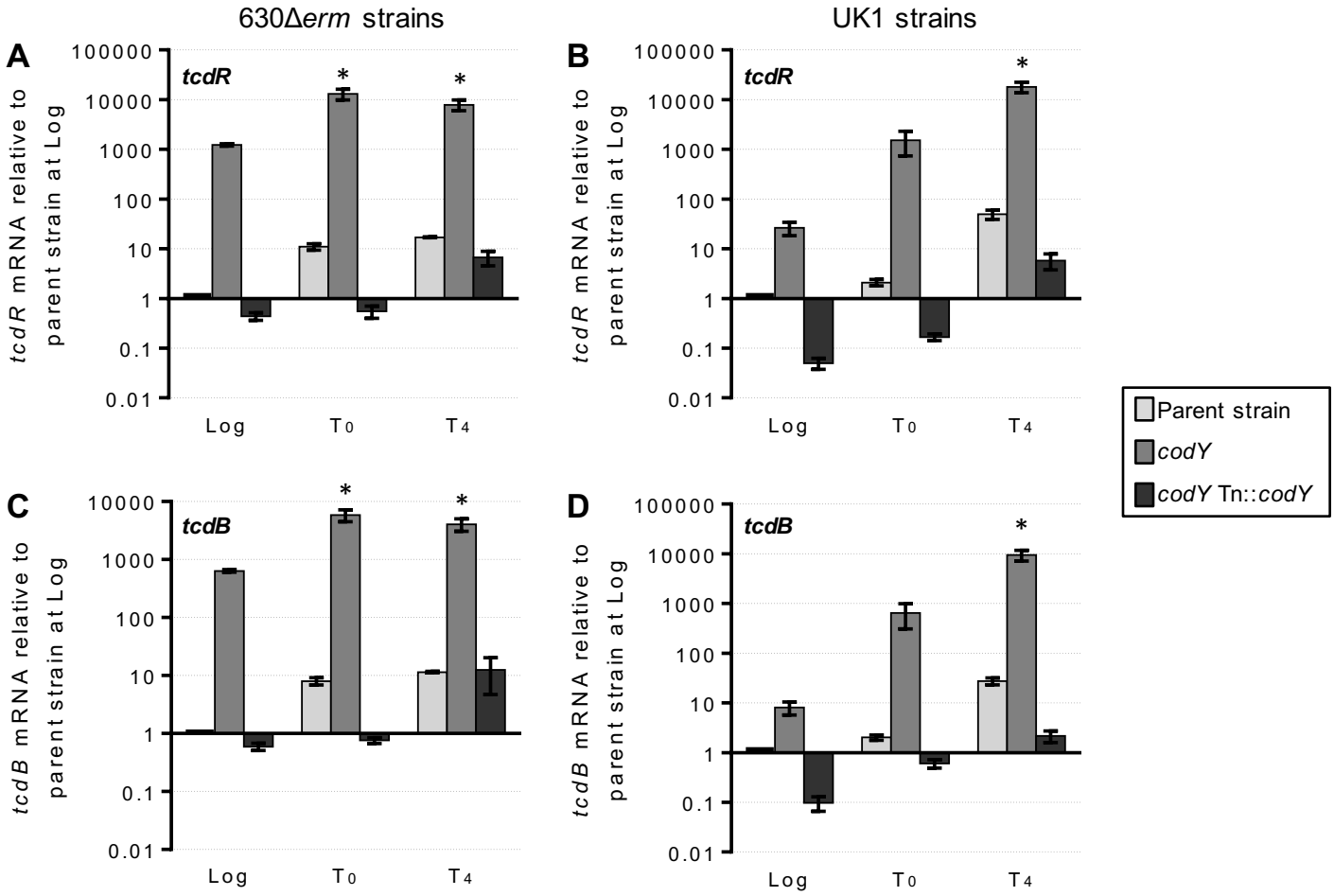

Figure S5. Expression of the toxin-specific sigma factor, *tcdR*, and toxin gene, *tcdB*, is increased in *codY* mutants. qRT-PCR analysis of *tcdR* and *tcdB* in (**A**,**C**) 630Δ*erm,* MC364 (630Δ*erm codY::ermB*), MC442 (630Δ*erm codY* Tn::*codY*) and (**B**,**D**) UK1, LB-CD16 (UK1 *codY::ermB*), and MC443 (UK1 *codY* Tn::*codY*) grown in 70:30 sporulation media. Samples for RNA were collected during logarithmic growth (OD_{600} ~0.5), transition to stationary phase (T_0), and four hours post the transition to stationary phase (T_4). The means and standard error of the means of four biological replicates are shown. A two-way repeated measures ANOVA followed by Dunnett's multiple comparison test was used to compare parent strain with the *codY* mutant or the parent strain with the respective complemented strain. *, $P \leq 0.05$.

Figure. S6

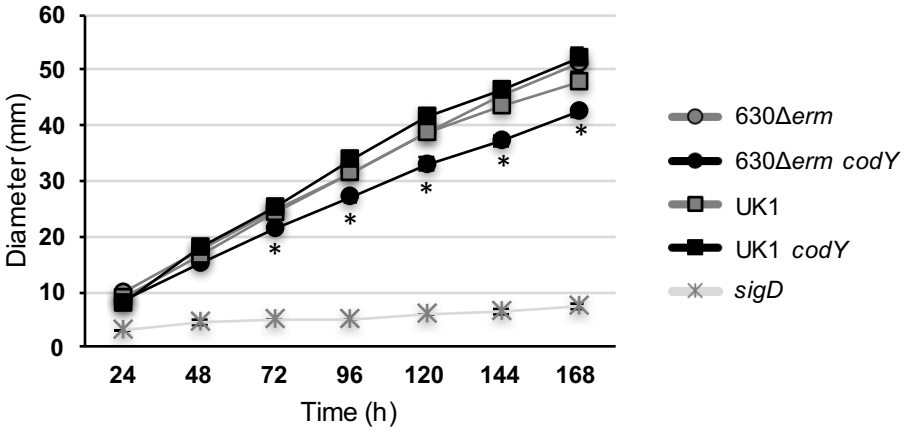

Figure S6. Motility phenotypes of *codY* mutants. (**A**) Motility of 630Δ*erm*, MC364 (630 *codY*), UK1, LB-CD16 (UK1 *codY*) and the non-motile strain, *sigD* (RT1075) on the surface of one-half concentration BHI with 0.3% agar plate. The diameters of motility (mm) were measured every 24 h for a total of 168 h. The means and SEM of four biological replicates are shown. *, $P \leq 0.05$ by a two-tailed Student's *t* test.

Fig. S7

A **P*sinR***

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-600  ATTAAATTAT TTTATAAGAT TATTACTCTA CTATAAATCT TGTATATAAC TTGTATTTAT AAGTATTTGT ATATTTATTT TTTATAATCT AAACTAATAT
-500  TATATATTTT CATTATTTTT TTAATTATAA TTTTTTTAAA ATTTTATTCT AAATGCCTTA CTTATAATT AATTTTTTAT TTCACCTATAT ATAATTAGAT
-400  TTAAAAATGT TTACCTTACC AATATAATGA TTAAACTCAA AAGTCTTATT GAAAAATTTT CTATTTACAT AAATTGTTCA ATATATAAAA TAGAAAATTT
-300  TTTTAATTTT CAAAATATAT TCTACATATC TAATATGTAA TTACAATAAA AAATGCTATA ATTAACATAT TATTAATTCG TTTTATTATT GTAGTATGGC
                 A
-200  TATGAAATTA TGAAAATATT TCTCTAATTA AAAATATGAT TTATCCATAT TTGTTTATTG TAGATACAGA CTATACAAAA ATAAATAGCA CTTATTTCAG
-100  TATAAAAACA TATATAGTCT ATATTTAGAC TAAAAAAGTA TAAATAGTCT ATATAAAACA ACTTAAACTA TGAATATAAT CTAAAGTGGA GGGATAATAA TTG
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B **P*oppB***

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-400  ACTGTGTACA TAGTTTTAGA ATAAAGTTCT TTTAAGAGCA TGGCTATTCT AAAACAGTCA TAATTACTTT TCAATAAAAT CACCTCTTAA ATGGTTTGCA
                                                        -250
-300  TACAAACTAA TATAATATAC TTTTCTTATA TAGTCAACCT AAAATTTTAT AATTTTATAG AAAATAATGA AGAATAGAAT ATAAAAATTT TATTTTAGGT
                                                                                          C
                                  -170                  -150
-200  ATAAATAAAA TAATTTGATG AAATTTTAAC AATTTTTAAA AAGTTTGTTT ACACAGTTAA TAAATGATGC TAAAATTAAC TCATAGATAA ATATAGAAAA
                          A
                                                                                  Δ
-100  TTTAATTTTG TTTAATGATA ATGGAACCAC GAAGGTTTTA ACGTTACTTT ACGTTTGCTT TTATGTGGTT TTTTTTATTG CAACAAAGGG GGGTTGGGGT TTG
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Figure S7. The promoter region of *sinR* and *oppB*. 600 bp DNA sequence upstream of the predicted *sinR* translational start site from the 630 genome (**A**). 400 bp DNA sequence upstream of the predicted *oppB* translational start site from the 630 genome (**B**). Sequence differences in the UK1 strain are noted above the 630 sequence. Δ denotes a deletion. Alterations made by site-directed mutagenesis are noted below the sequence. The putative translation start site is marked by a dashed underline. The previously identified CcpA (67) and CodY (32, 37) binding sites are marked by a solid underline and blue font, respectively. The evaluated promoter fusion lengths are indicated by bolded markers.