**Supporting information**

**Fig. S1.** RNA-Seq analyses of *spoIID*, *spoIIP*, and *spoIIM* transcription in sporulating JIR8094 strains visualized using the Integrated Genome Viewer software

**Fig. S2.** Construction of mutants lacking engulfment-related genes.

**Fig. S3.** Comparison of heat-resistant spore formation by Δ*IIQ* grown in broth vs. on plates.

**Fig. S4.** Read-through transcription of *spoIIP* increases IIP levels in *C. difficile*.

**Fig. S5.** Fluorescence microscopy analyses of engulfment progression.

**Fig. S6.** Severe engulfment and coat localization defects in Δ*IID*, Δ*IIP*, Δ*IIM* and Δ*IIQ* mutant derivatives.

**Fig. S7.** Complementation of Δ*sipL* by a construct encoding SipL-mCherry.

**Fig. S8.** Western blot analyses of mCherry fusion proteins used in localization studies.

**Fig. S9.** Localization of the outer basement layer protein, SipL, in engulfment mutants.

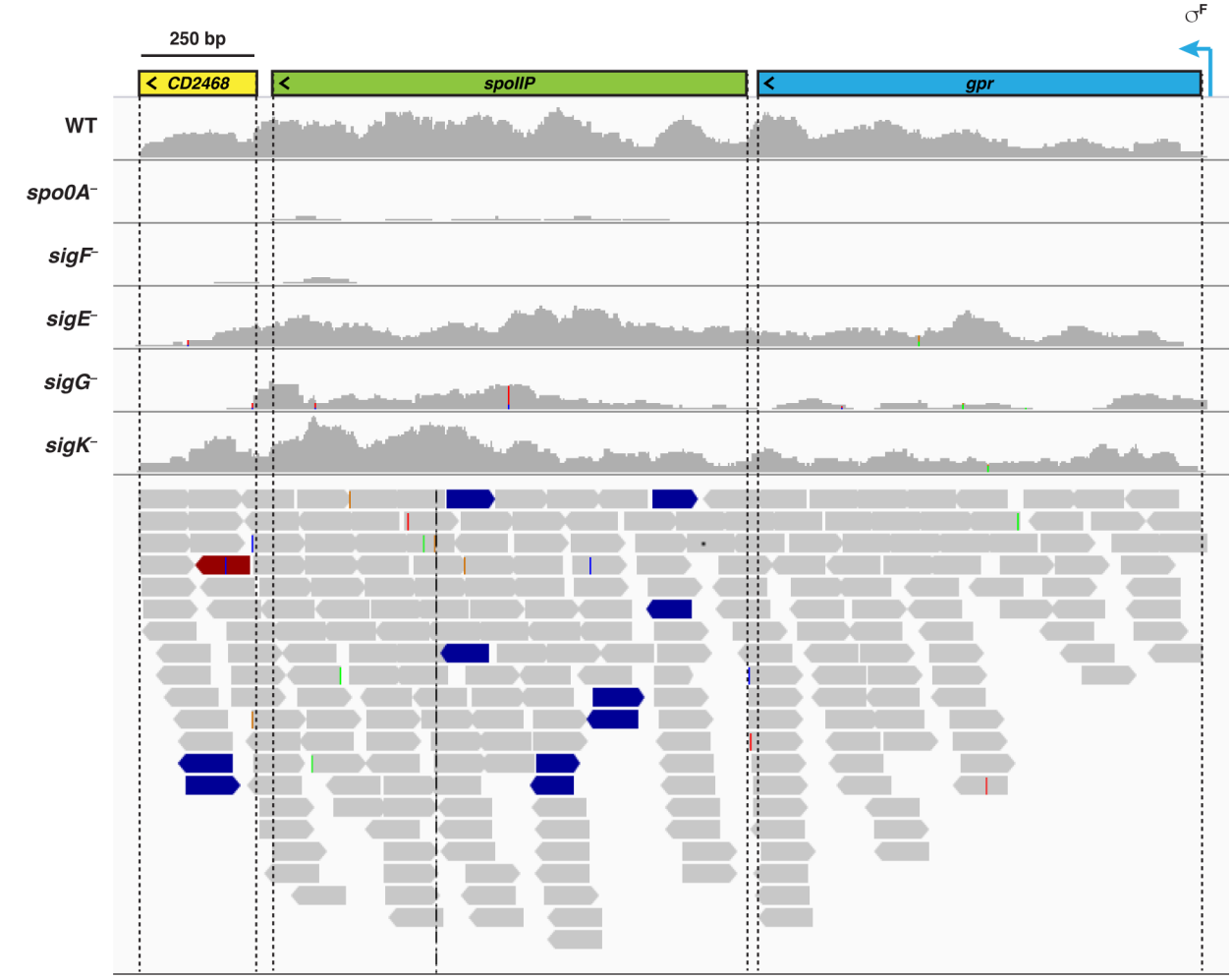
**Supplementary Text S1** – Description of cloning of plasmid constructs.

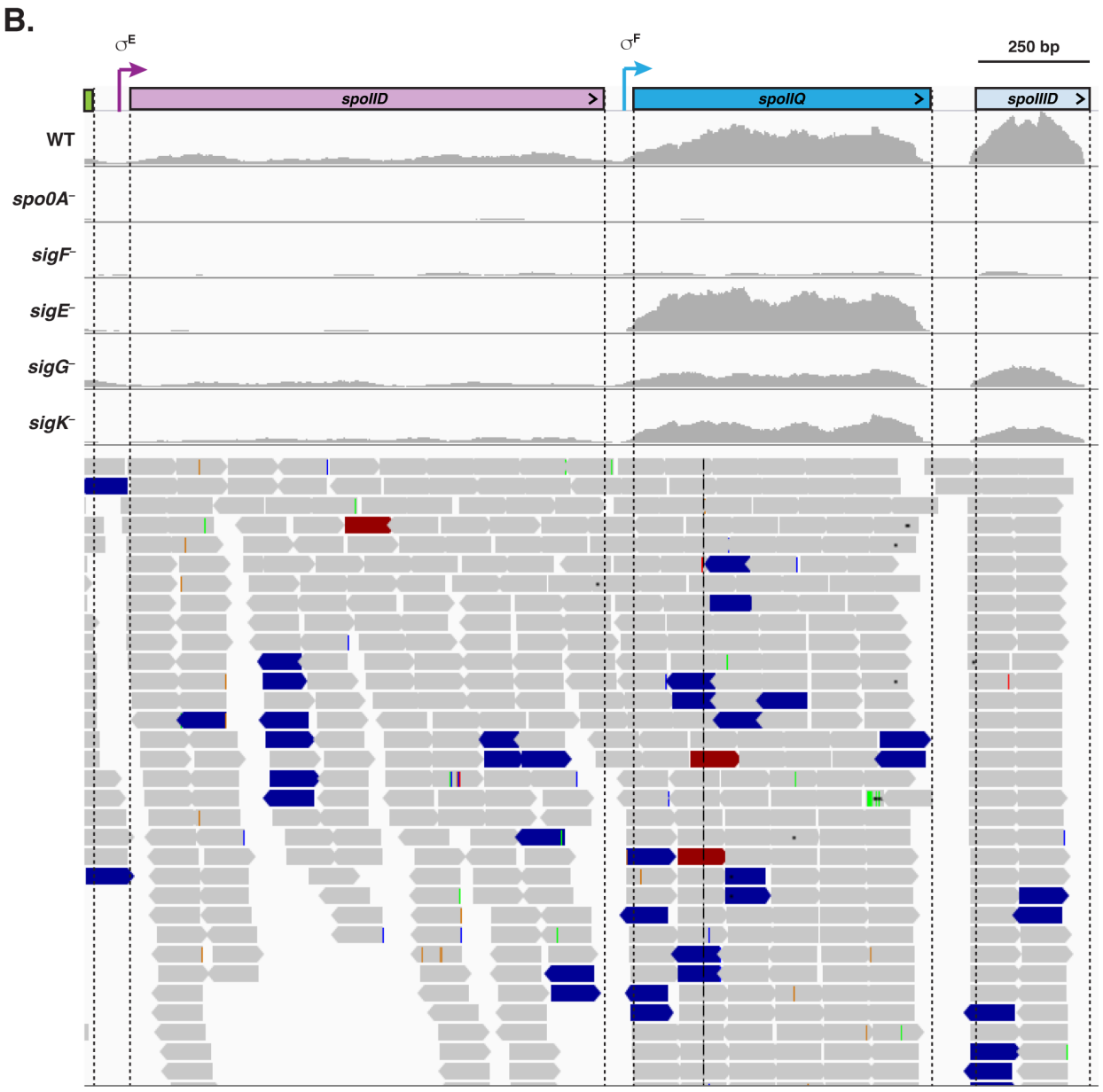
**Supplementary Table S1** – *C. difficile* and *E. coli* strains used in this study.

**Supplementary Table S2** – Primers used in this study.

**Supplementary Table S3 -** Sporulation heat resistance ratios determined for all strains across all replicates.

**A.**




B.
σE
σF
250 bp
spoIID
spoIIQ
spoIIID
WT
spo0A⁻
sigF⁻
sigE⁻
sigG⁻
sigK⁻

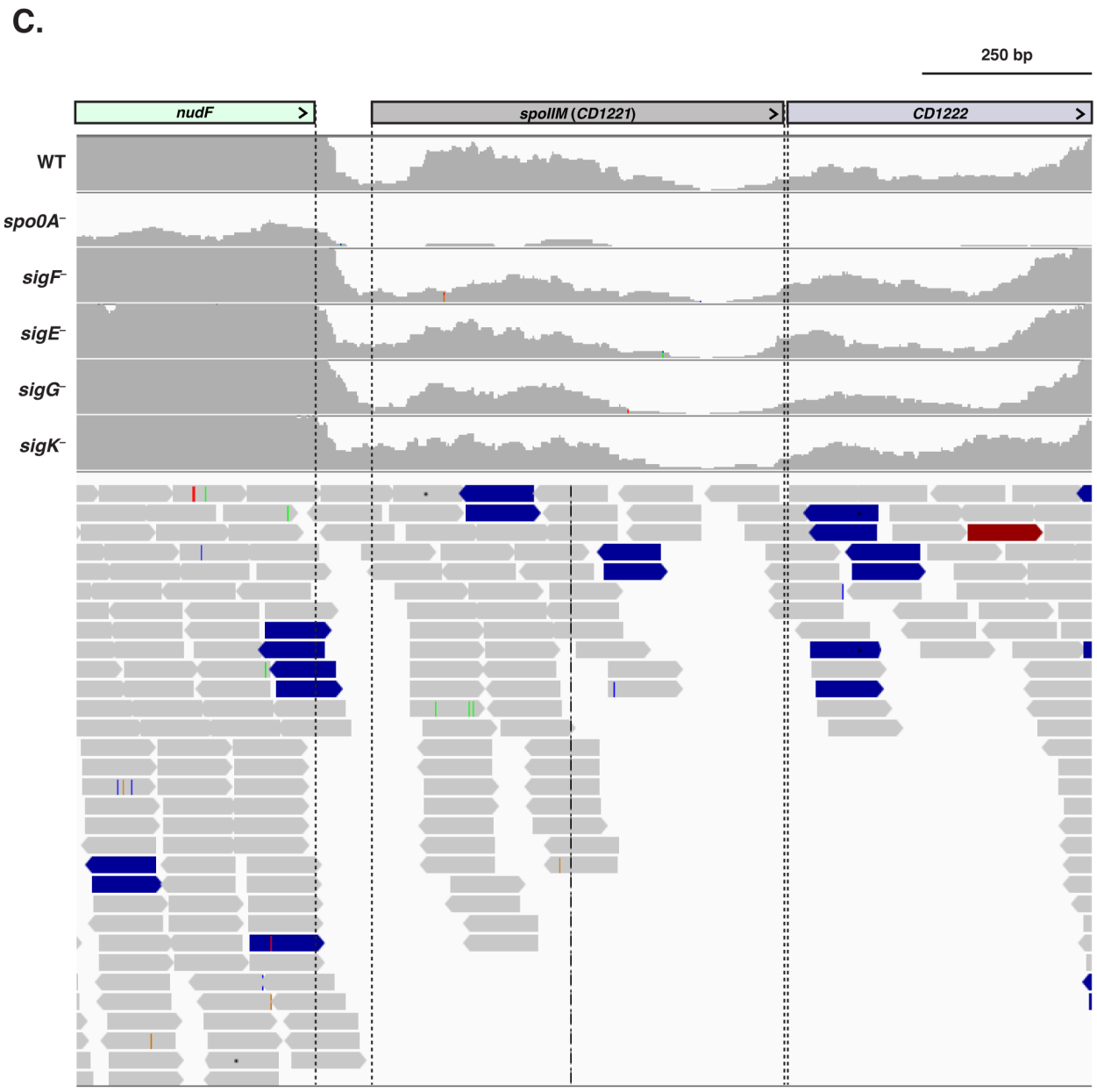



**Fig. S1. RNA-Seq analyses of *spoIID*, *spoIIP*, and *spoIIM* transcription in sporulating JIR8094 strains visualized using the Integrated Genome Viewer software** (Robinson *et al.*, 2011). Histograms of RNA sequence reads (grey) in wildtype JIR8094 and sporulation mutants created using Targetron-based gene disruption (Heap *et al.*, 2007). The reads mapped to these loci are shown below the histograms. *spo0A* encodes a master transcriptional activator required for inducing sporulation; *sigF* encodes $\sigma^F$, a forespore-specific sigma factor that is necessary for

activating the forespore-specific sigma factor, $\sigma^G$, and contributes to the activation of $\sigma^E$, a mother cell-specific sigma factor encoded by *sigE* (Fimlaid & Shen, 2015). $\sigma^E$ is necessary for transcriptional activation of *sigK*, which encodes the mother cell-specific sigma factor, $\sigma^K$ (Pishdadian *et al.*, 2015, Saujet *et al.*, 2013). Genes are shown as colored rectangles, with the chevron indicating the direction of transcription. Bent arrows represent transcriptional start sites. Promoters whose sporulation sigma factor regulation had been previously established are shown (Saujet *et al.*, 2013). (A) *spoIIP* is encoded downstream of *gpr*; (B) *spoIID* is encoded upstream of *spoIIQ*; and (C) *spoIIM* (*CD1221*) is encoded upstream of a XerC recombinase gene. The histograms shown are representative of the results obtained from three independent biological replicates.

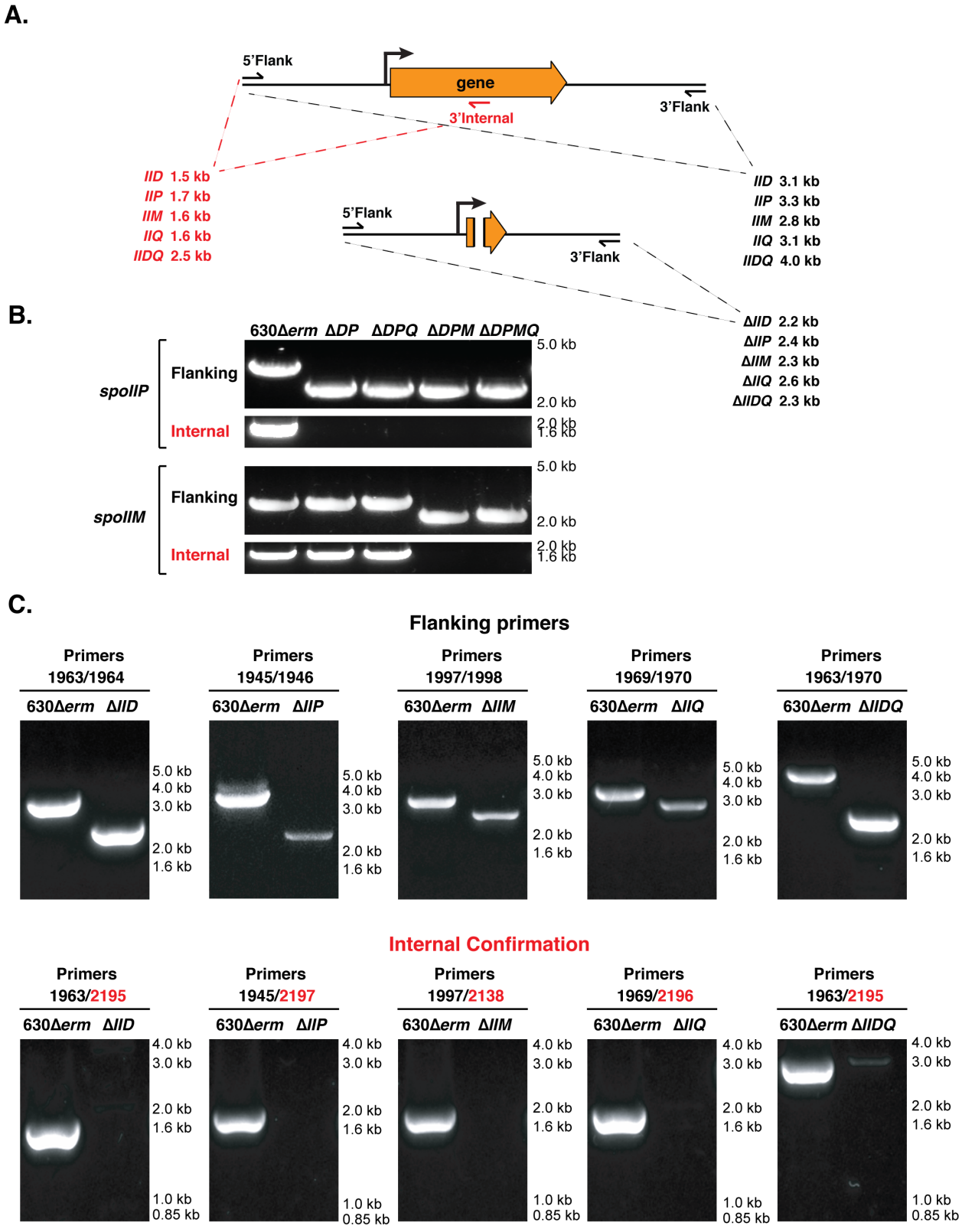



**Fig. S2. Construction of mutants lacking engulfment-related genes.** (A) Schematic of gene

deletions. Flanking primers bind outside the region of homology cloned into pMTL-YN3 and were used, along with an internal red primer, to confirm the deletion of *IIP* and *IIM* in multi-gene deletion strains (B). (C) Colony PCR of first generation single mutants, Δ*IID*, Δ*IIP*, Δ*IIM*, and Δ*IIQ*, and the Δ*IIDQ* double mutant using either the flanking primer pair or the 5' flanking primer with the 3' internal primer to assess strain purity.

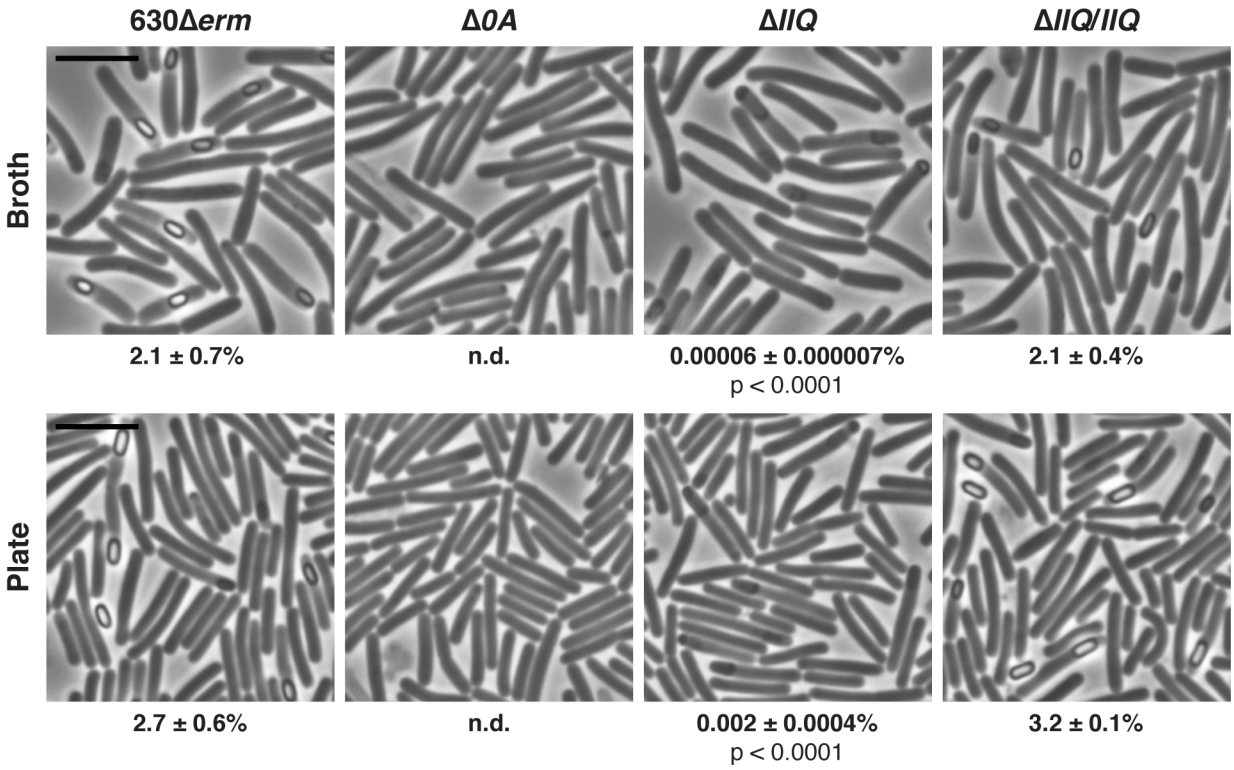



**Fig. S3. Comparison of heat-resistant spore formation by Δ*IIQ* grown in broth vs. on plates.** Phase-contrast microscopy analyses of the indicated strains after 24 hrs of sporulation. The ratio of heat-resistant spores measured for each strain from 23 hr sporulating cultures was determined based on three independent biological replicates. Statistical significance relative to wild type was determined using a one-way ANOVA and Tukey's test. Scale bars represent 5 μm. The limit of detection of the assay is $10^{-6}$.

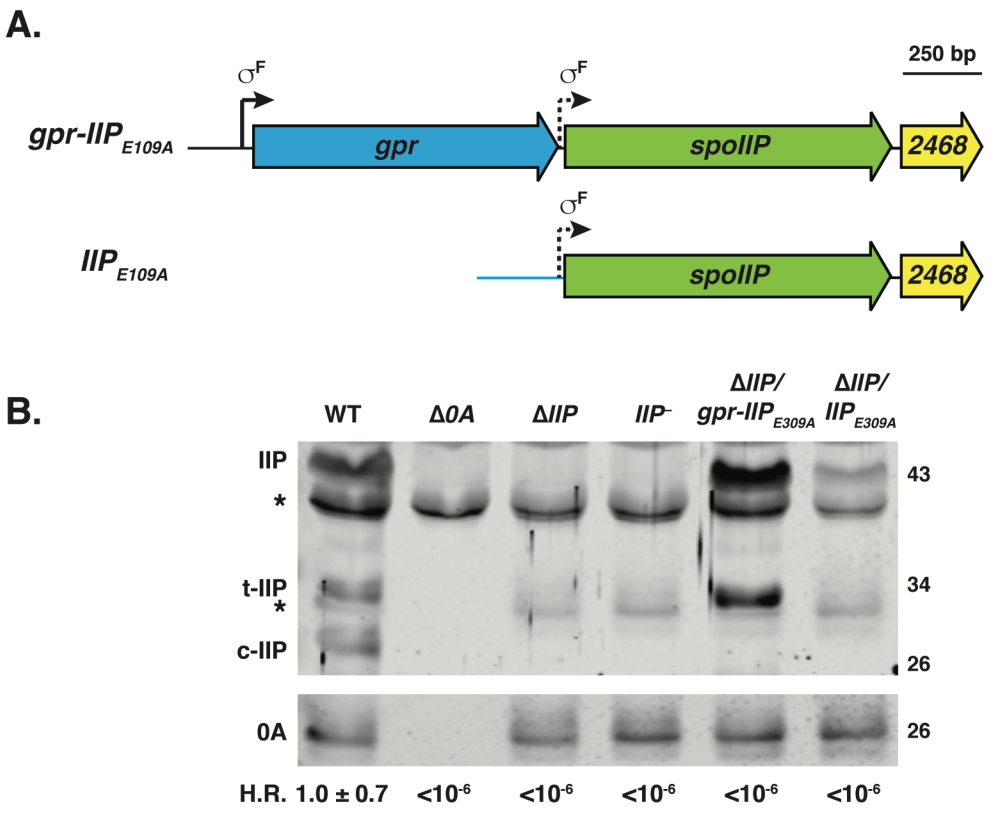



**Fig. S4. Read-through transcription of *spoIIP* increases IIP levels in *C. difficile*.** (A) Schematic of *spoIIP* complementation constructs encoding Glu309A mutations in IIP. Bent arrows represent transcriptional start sites, although only the *gpr* transcriptional start site has been mapped; dashed lines indicate likely position of the proximal promoter based on results of this figure. (B) Western blot analyses of IIP in Δ*spoIIP* complementation constructs with *spoIIP* deletion and targetron (*IIP*$^{-}$) mutant controls. Three separate isoforms were detected for IIP in wild type: full-length (IIP), truncated IIP (t-IIP), and cleaved IIP (c-IIP). The predicted MW of IIP is 38 kDa, and the MW of IIP lacking its signal peptide is 35 kDa. The asterisks denote non-specific proteins recognized by the polyclonal anti-IIP antibodies. Spo0A was used as a proxy for measuring sporulation induction (Dembek *et al.*, 2017, Putnam *et al.*, 2013). The western blots shown are representative of the results from three independent biological replicates. Heat resistance (H.R.) efficiencies were determined from 23 hr cultures and represent the mean and

standard deviation for a given strain relative to wild type based on a minimum of three independent biological replicates. The limit of detection of the assay is $10^{-6}$.

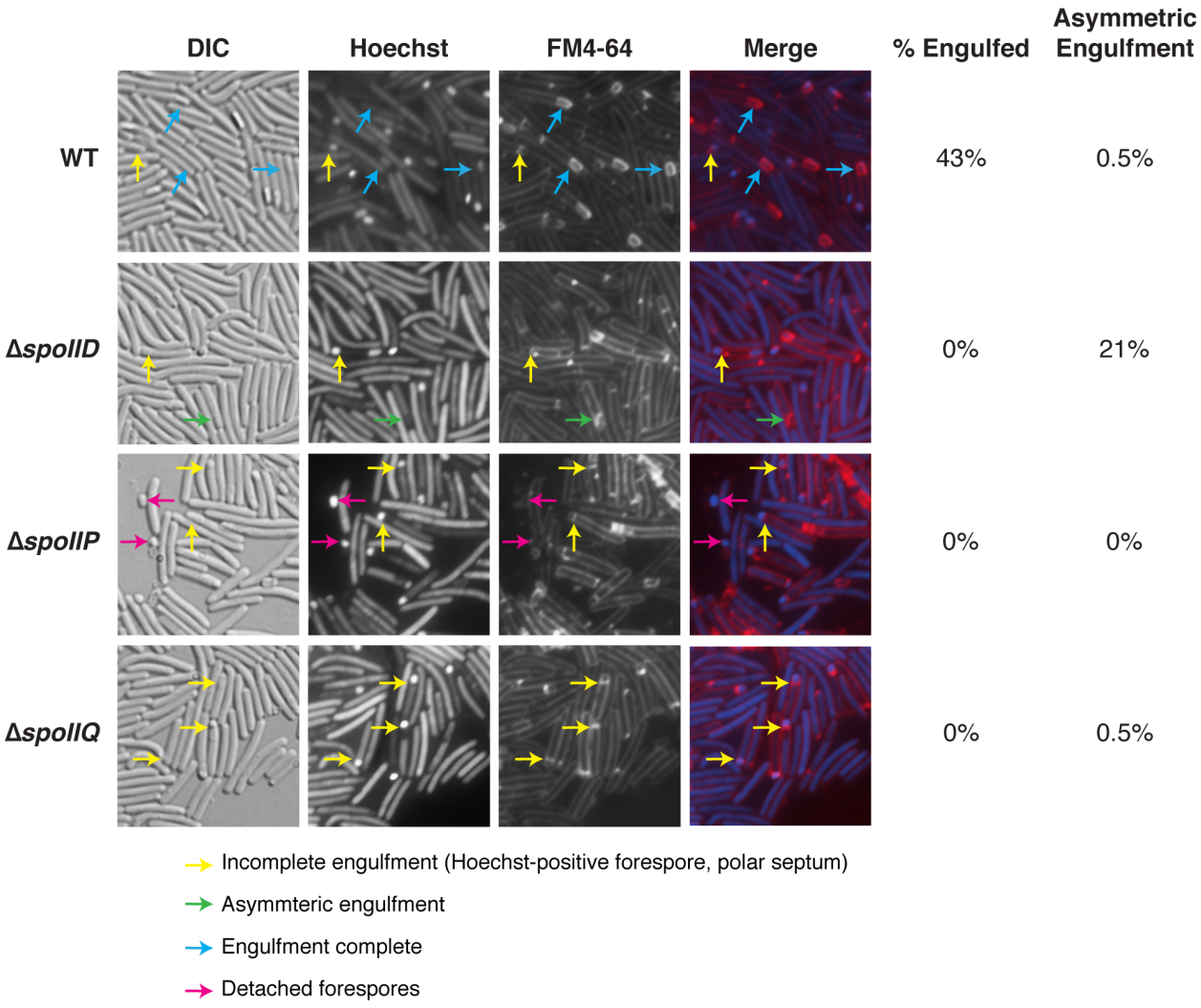
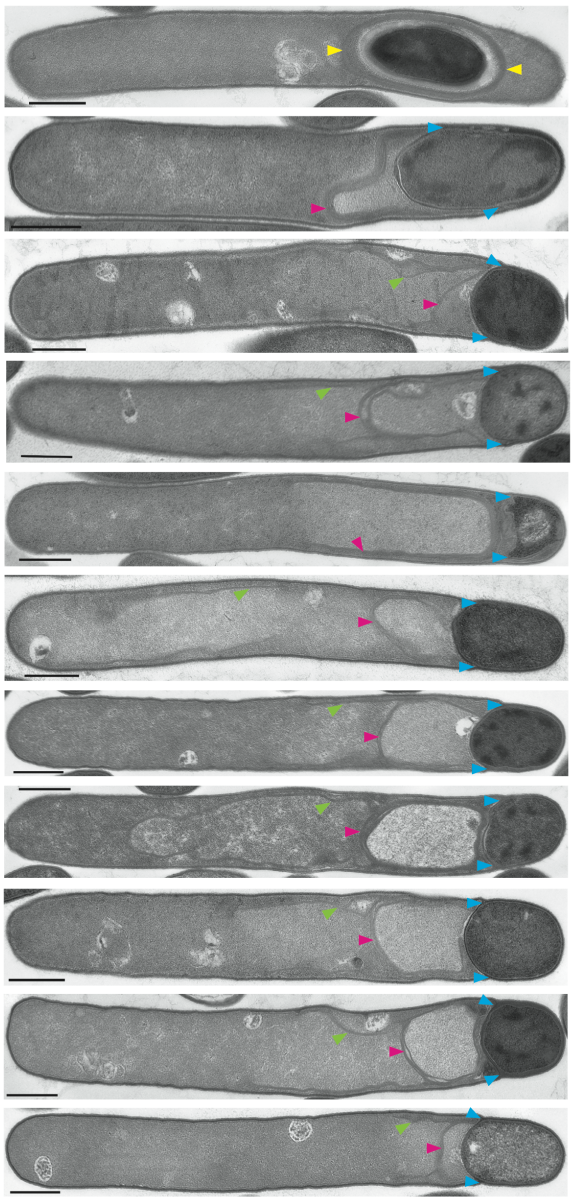



**Fig. S5. Fluorescence microscopy analyses of engulfment**. Wild type and single gene engulfment mutants were grown on sporulation media for 24 hrs and evaluated by live differential interference contrast (DIC) and fluorescence microscopy. Hoechst was used to stain the nucleoid (blue), and FM4-64 was used to stain membranes (red). Fully engulfed cells exclude the Hoechst stain from the forespore (Fimlaid *et al.*, 2015, Sharp & Pogliano, 1999), while engulfed cells that complete membrane fission exclude FM4-64 (Doan *et al.*, 2013). Yellow arrows designate cells that have completed engulfment, green arrows highlight forepores undergoing asymmetric engulfment, and pink arrows indicate detached forespores observed in Δ*IIP*. Sporulation was arrested at asymmetric division in Δ*IIP*, while Δ*IIQ* and Δ*IID* forespores

exhibited greater curvature of the forespore membrane; Δ*IID* forespores exhibited asymmetric engulfment at higher frequencies than wild type. The percentage of cells that completed engulfment or exhibited asymmetric engulfment is shown based on analyses of >200 cells.



| | Engulfed | Coat Bearding | Double Beard | Cytosolic |
|---|---|---|---|---|
| WT | 100% | 0% | 0% | 0% |
| Δ*IID* | 0% | 86% | 0% | 4% |
| Δ*IIP* | 0% | 82% | 32% | 12% |
| Δ*IIM* | 86% | 16% | 2% | 4% |
| Δ*IIQ* | 2% | 70% | 12% | 22% |
| Δ*IIDQ* | 0% | 70% | 18% | 30% |
| Δ*IIDP* | 0% | 82% | 16% | 14% |
| Δ*IIPQ* | 0% | 73% | 29% | 40% |
| Δ*IIDPQ* | 0% | 80% | 34% | 20% |
| Δ*IIDPM* | 0% | 72% | 24% | 22% |
| Δ*IIDPMQ* | 0% | 72% | 34% | 26% |

Properly localized coat
Mislocalized coat (bearding)
Double beard
Leading edge of engulfment

**Fig. S6. Severe engulfment and coat localization defects in Δ*IID*, Δ*IIP*, Δ*IIM* and Δ*IIQ* mutant derivatives.** Transmission electron microscopy (TEM) analyses of wildtype 630Δ*erm* and engulfment mutants after 23 hrs of sporulation induction. Scale bars represent 500 nm. Yellow arrows mark properly localized coat, and pink arrows mark mislocalized coat. Blue arrows mark the leading edge of the engulfing membranes in cells that fail to complete engulfment. The percentages shown are based on analyses of 50 cells for each strain with visible signs of sporulation from the same single biological replicate shown in Fig. 4 with the exception of Δ*IIPQ*. Coat bearding refers to polymerized coat that sloughs off from the forespore. "Double Beard" refers to when two layers of polymerized coat were observed such as in the Δ*IIDPM* and Δ*IIDPMQ* mutants (green arrows). Cytosolic refers to polymerized coat visible in the mother cell cytosol, i.e. detached from the forespore.

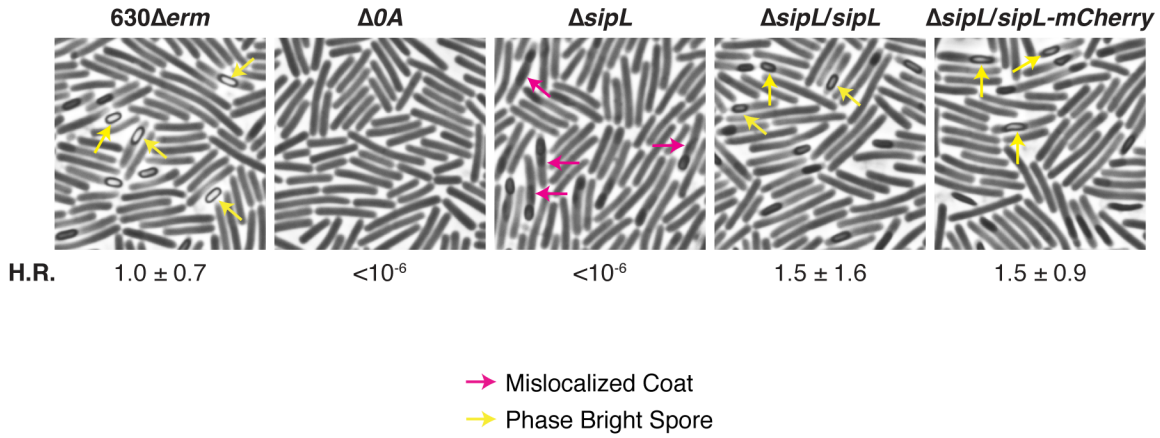



**Fig. S7. Complementation of Δ*sipL* with a *sipL-mCherry* construct.** Phase-contrast microscopy analyses of wild type 630Δ*erm*, Δ*spo0A* (Δ*0A*), and Δ*sipL* strains 21 hrs after sporulation induction. Yellow arrows mark example phase-bright forespores, while pink arrows demarcate regions suspected to be mislocalized coat based on previous studies (Fimlaid *et al.*, 2015, Ribis *et al.*, 2017). Heat resistance (H.R.) efficiencies were determined from 23 hr cultures and represent the mean and standard deviation for a given strain relative to wild type based on a minimum of three independent biological replicates. The limit of detection of the assay is $10^{-6}$.

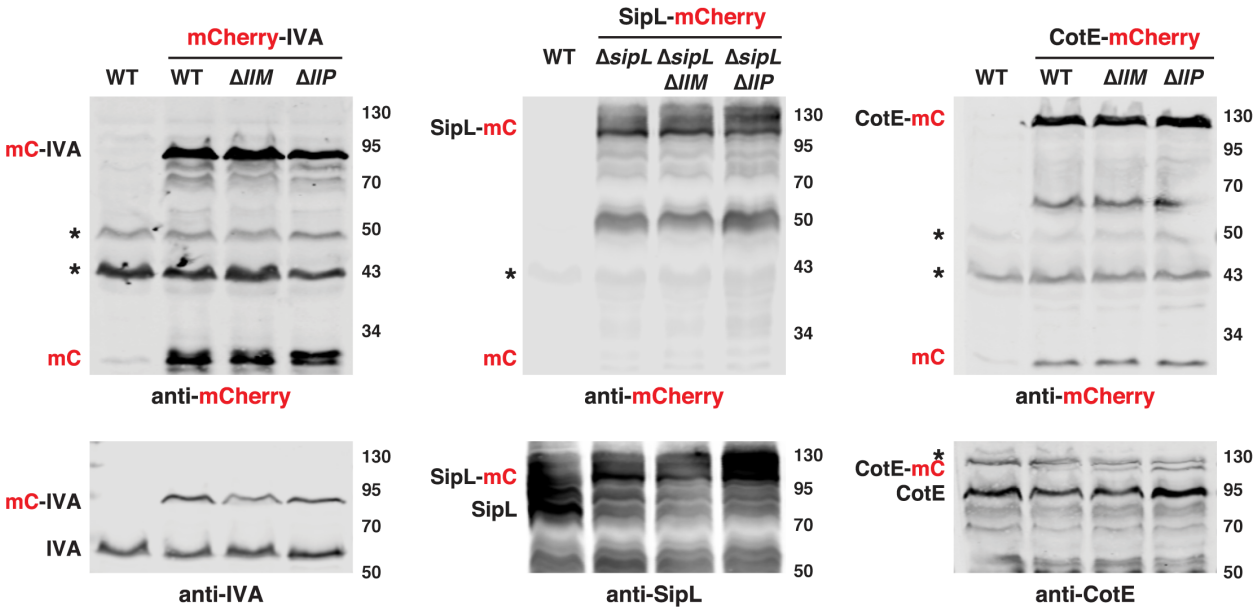



**Fig. S8. Western blot analyses of mCherry fusion proteins used in localization studies**. Antibodies to mCherry (mC), IVA, SipL and CotE were used as indicated. Asterisks indicate non-specific bands. Partial processing of mCherry-IVA releases free mCherry as previously reported (Ribis *et al.*, 2017). Several isoforms of SipL and SipL-mCherry are detected, most that run higher than expected. The western blots shown are representative of the results from three independent biological replicates.

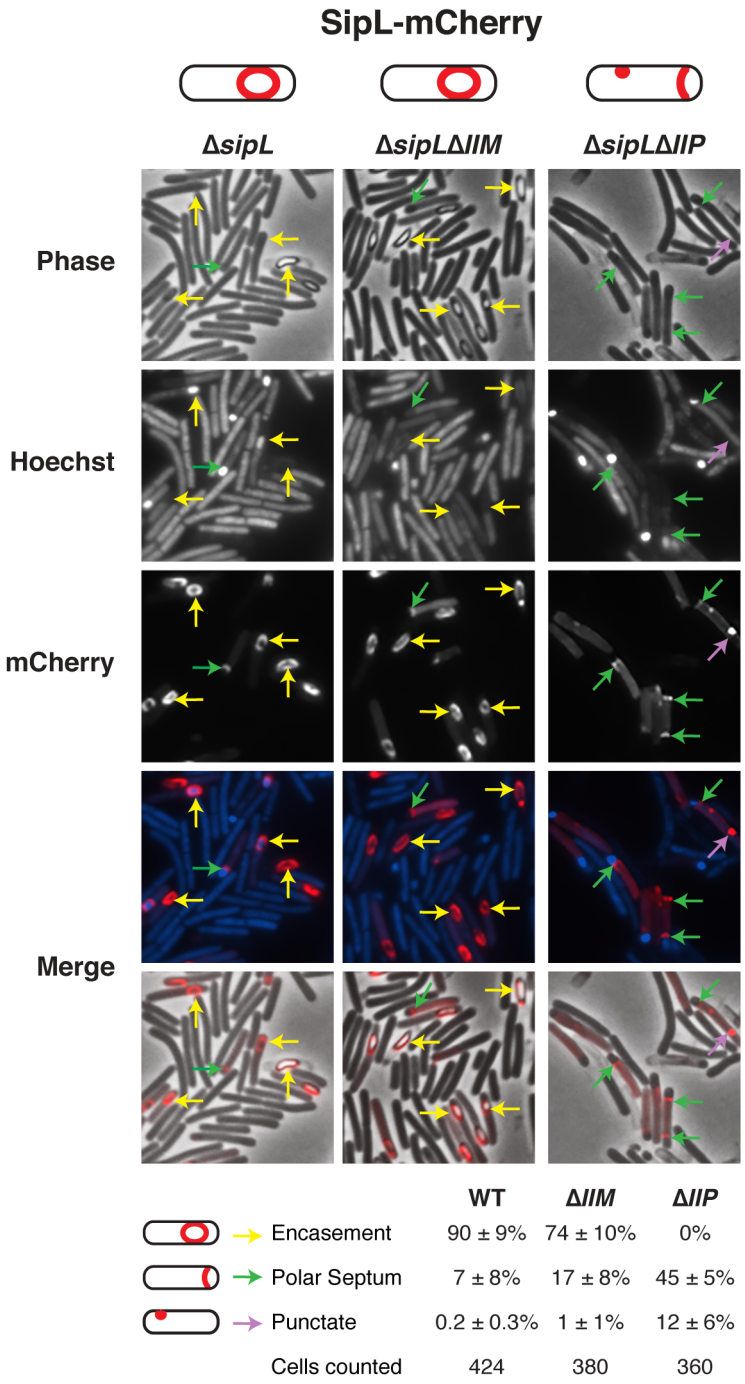



**Fig. S9. Localization of the outer basement layer protein, SipL, in engulfment mutants**. Fluorescence microscopy analyses of Δ*sipL*, Δ*sipL*Δ*IIM*, and Δ*sipL*Δ*IIP* cells producing SipL-mCherry fusions at 23 hrs post sporulation induction. Phase-contrast (phase) microscopy was used to visualize sporulating cells. Hoechst staining of the nucleoid is shown in blue, and

mCherry fluorescence is shown in red. Engulfment completion excludes Hoechst from the forespore (Pogliano *et al.*, 1999). The merge of Hoechst and mCherry (top) and phase-contrast and mCherry (bottom) is shown. Yellow arrows denote encasement of the forespore; green arrows highlight staining along the presumed polar septum based on Hoechst labeling; and purple arrows highlight punctate foci. Schematics depicting the primary localization pattern of SipL-mCherry, and the percentage of a given phenotype is shown as is the total number of cells analyzed.

# Supplementary Text S1: Cloning of plasmid constructs

## 1. Deletion constructs

**pMTL-YN3 Δ*IID***. Primer pair #1959 and 1961 was used to amplify the region 940 bp upstream of *spoIID* primer pair #1960 and 1962 to amplify the region 921 bp down stream of *spoIID*. The resulting PCR products were used in a PCR SOE (Horton *et al.*, 1989) with the flanking primers #1959 and 1962 to generate a fragment than encodes an in-frame deletion of *spoIID* where the first 12 codons are linked to the last 22 codons. The PCR SOE fragment was digested with AscI and SbfI and ligated to pMTL-YN3 digested with the same enzymes.

**pMTL-YN3-Δ*IIP***. Primer pair #1941 and 1943 was used to amplify the region 1000 bp upstream of *spoIIP*; primer pair #1942 and 1944 was used to amplify the region 1000 bp downstream of *spoIIP*. The resulting PCR products were used in a PCR SOE with the flanking primers #1941 and 1944 to generate a fragment than encodes an in-frame deletion of *spoIIP* where the first 21 codons are linked to the last 12 codons. The PCR SOE fragment was digested with AscI and SbfI and ligated to pMTL-YN3 digested with the same enzymes.

**pMTL-YN3-Δ*IIM***. Primer pair #1999 and 2002 was used to amplify the region 1001 bp upstream of *spoIIM* primer pair #2001 and 2000 to amplify the region 1059 bp downstream of *spoIIM*. The resulting PCR products were used in a PCR SOE with the flanking primers #1999 and 2000 to generate a fragment than encodes an in-frame deletion of *spoIIM* where the first 22 codons are linked to the last 11 codons. The PCR SOE fragment was digested with AscI and SbfI and ligated to pMTL-YN3 digested with the same enzymes.

**pMTL-YN3-Δ*IIQ***. Primer pair #1965 and 1967 was used to amplify the region 1025 bp upstream of *spoIIQ* primer pair #1966 and 1968 to amplify the region 975 bp downstream of *spoIIQ*. The resulting PCR products were used in a PCR SOE with the flanking primers #1965 and 1968 to generate a fragment than encodes an in-frame deletion of *spoIIQ* where the first 28 codons are linked to the last 22 codons. The PCR SOE fragment was digested with AscI and SbfI and ligated to pMTL-YN3 digested with the same enzymes.

**pMTL-YN3-Δ*IIDQ***. Primer pair #1959 and 1974 was used to amplify the region 1025 bp upstream of *spoIIDQ* primer pair #1973 and 1968 to amplify the region 975 bp downstream of *spoIIDQ*. The resulting PCR products were used in a PCR SOE with the flanking primers #1959 and 1968 to generate a fragment than encodes an in-frame deletion of *spoIIDQ* where the first 28 codons of *spoIID* are linked to the last 22 codons of *spoIIQ*. The PCR SOE fragment was digested with AscI and SbfI and ligated to pMTL-YN3 digested with the same enzymes.

**pMTL-YN3-Δ*sipL***. Primer pair #1995 and 1760 was used to amplify the region 999 bp upstream of *sipL.* primer pair #1759 and 2088 to amplify the region 591 bp down stream of *sipL*. The resulting PCR products were cloned into pMTL-YN3 digested with AscI and SbfI using Gibson assembly (Gibson *et al.*, 2009). The construct encodes an in-frame deletion of *sipL* where the first 15 codons of *sipL* are linked to the last 15 codons.

## 2. Targetron construct

To create the *spoIIP* targetron construct, a modified plasmid containing the retargeting group II intron, pCE245 (a gift from C. Ellermeier, University of Iowa) was used as a template. Primers #1487, 1488, 1489, and 532 (EBS universal primer, Sigma Aldrich) were used.

## 3. Complementation constructs

**pMTL-YN1C-*spoIID***. Primer pair #1572 and 1573 were used to amplify the region spanning 246 bp upstream of *spoIID* and 35 bp downstream of *spoIID*. The resulting product was digested with NotI and XhoI then ligated into pMTL-YN1C digested with the same enzymes.

**pMTL-YN1C-*spoIID*$_{E101A}$**. Primer pair #2231 and 2380 and primer pair #2379 and 2378 were used to amplify regions spanning 246 bp upstream of *spoIID* and 35 bp downstream of *spoIID*. Primers 2379 and 2380 encode a Glu309Ala mutation in *spoIIP* that inactivates its catalytic function. The PCR products were cloned into pMTL-YN1C digested with NotI and XhoI using Gibson assembly.

**pMTL-YN1C-*spoIIP*$_{E309A}$**. Primer pair #2481 and 2382 and primer pair #2381 and 2383 were used to amplify regions spanning 269 bp upstream of *spoIIP* and 282 bp downstream of *spoIIP* (includes *CD630_24860*). Primers 2382 and 2383 encode a Glu309Ala mutation in *spoIIP* that inactivates its catalytic function. The PCR products were cloned into pMTL-YN1C digested with NotI and XhoI using Gibson assembly.

**pMTL-YN1C-*gpr-spoIIP*$_{E309A}$**. Primer pair #2352 and 2382 and primer pair #2381 and 2383 were used to amplify regions spanning 204 bp upstream of *gpr* and 282 bp downstream of *spoIIP* (includes *CD630_24860*). Primers 2382 and 2383 encode a Glu309Ala mutation in *spoIIP* that inactivates its catalytic function. The PCR products were cloned into pMTL-YN1C digested with NotI and XhoI using Gibson assembly.

**pMTL-YN1C-*spoIIQ***. Primer pair #1177 and 1178 were used to amplify the region spanning 106 bp upstream of *spoIIQ* and 9 bp downstream of *spoIIQ*. The resulting product was digested with NotI and XhoI then ligated into pMTL-YN1C digested with the same enzymes.

**pMTL-YN1C-*spoIIQ*$_{H120A}$**. Primer pair #2449 and 1851 and primer pair #1850 and 2232 were used to amplify regions spanning 106 bp upstream of *spoIIQ* and 9 bp downstream of *spoIIQ* (includes *CD630_24860*). Primers 2382 and 2383 encode a His120Ala mutation in *spoIIQ* that disrupts binding to $Zn^{2+}$ and would inactivate IIQ's putative endopeptidase activity. The PCR products were cloned into pMTL-YN1C digested with NotI and XhoI using Gibson assembly.

**pMTL-YN1C-*spoIID-spoIIQ***. Primer pair #2231 and 2232 were used to amplify the region spanning 246 bp upstream of *spoIID* and 9 bp downstream of *spoIIQ*. The resulting product was cloned into pMTL-YN1C digested with NotI and XhoI using Gibson assembly.

**pMTL-YN1C-*spoIIM***. Primer pair #1576 and 1577 were used to amplify the region spanning 231 bp upstream of *spoIIM* and 82 bp downstream of *spoIIM*. The resulting product was digested with NotI and XhoI then ligated into pMTL-YN1C digested with the same enzymes.

**pMTL-YN1C-*sipL***. Plasmid pMTL83151-*sipL* (Putnam *et al.*, 2013) was digested with NotI/XhoI, and the resulting *sipL* fragment (include 318 bp upstream and 102 bp downstream of *sipL* was gel-purified and ligated into pMTL-YN1C digested with the same enzymes.

## 4. mCherry constructs

**pMTL-YN1C-*sipL-mCherry***. Primer pair #2165 and 2212 was used to amplify the *sipL* gene including 318 bp of its upstream region. Primer pair #2211 and 2133 was used to amplify the *mCherry* gene with an alanine codon linker using the codon-optimized *mCherry* construct from Ransom *et al*. as the template (Ransom *et al.*, 2015). The PCR products were cloned into pMTL-YN1C digested with NotI and XhoI using Gibson assembly.

**pMTL-YN1C-*cotE-mCherry***. Primer pair #2268 and 2264 was used to amplify the *cotE* gene including 228 bp of its upstream region. Primer pair #2263 and 2133 was used to amplify the *mCherry* gene with an alanine codon linker using the codon-optimized *mCherry* construct from **Ransom** *et al*. as the template. The PCR products were cloned into pMTL-YN1C digested with NotI and XhoI using Gibson assembly.

## 5. pET expression constructs

**pET21a-*spoIID*$_{\Delta 27aa}$.** Primer pair #1631 and 1632 were used to amplify *spoIID* lacking its first 27 codons and its stop codon. The resulting product was digested with NheI and XhoI then ligated into pET21a digested with the same enzymes.

**pET28a-*spoIIP*$_{\Delta 27aa}$.** Primer pair #2297 and 2316 were used to amplify *spoIIP* lacking its first 27 codons and its stop codon. The resulting product was cloned into pET28a digested with NcoI and XhoI using Gibson assembly.

## Supplementary Table S1. Strains used in this study

| Strain # | Strain name | Relevant genotype or features | Source/reference |
|---|---|---|---|
| ***C. difficile* strains – 630Δ*erm*** | | | |
| 756 | 630Δ*erm*Δ*pyrE* | *erm*-sensitive derivate of 630 with a deletion in *pyrE* | (Ng *et al.*, 2013) |
| 807 | 630Δ*erm*Δ*pyrE* Δ*IIP* | 630Δ*erm*Δ*pyrE* with *spoIIP* (*CD2469*) deleted | This study |
| 817 | 630Δ*erm*Δ*pyrE* Δ*IID* | 630Δ*erm*Δ*pyrE* with *spoIID* (*CD0126*) deleted | This study |
| 819 | 630Δ*erm*Δ*pyrE* Δ*IIQ* | 630Δ*erm*Δ*pyrE* with *spoIIQ* (*CD0125*) deleted | This study |
| 826 | 630Δ*erm*Δ*pyrE* Δ*IIDQ* | 630Δ*erm*Δ*pyrE* with *spoIID-spoIIQ* deleted | This study |
| 834 | 630Δ*erm* Δ*IIQ*/*IIQ* | 630Δ*erm*Δ*IIQ* with *spoIIQ* in the *pyrE* locus | This study |
| 838 | 630Δ*erm* Δ*IID*/*IID* | 630Δ*erm*Δ*IID* with *spoIID* in the *pyrE* locus | This study |
| 846 | 630Δ*erm*-p | *erm*-sensitive derivate of 630 with *pyrE* restored | (Donnelly *et al.*, 2017) |
| 849 | 630Δ*erm* Δ*spo0A*-p | 630Δ*erm*Δ*spo0A* with *pyrE* restored | (Donnelly *et al.*, 2017) |
| 854 | 630Δ*erm*Δ*pyrE* Δ*IIDPQ* | 630Δ*erm*Δ*pyrE* with *spoIID*, *spoIIP*, and *spoIIQ* deleted | This study |
| 907 | 630Δ*erm*Δ*pyrE* Δ*IIM* | 630Δ*erm*Δ*pyrE* with *spoIIM* (*CD1221*) deleted | This study |
| 910 | 630Δ*erm* Δ*IID*-p | 630Δ*erm*Δ*IID* with *pyrE* restored | This study |
| 913 | 630Δ*erm* Δ*IIP*-p | 630Δ*erm*Δ*IIP* with *pyrE* restored | This study |
| 916 | 630Δ*erm* Δ*IIQ*-p | 630Δ*erm*Δ*IIQ* with *pyrE* restored | This study |
| 919 | 630Δ*erm* Δ*IIDQ*-p | 630Δ*erm*Δ*IID*Δ*IIQ* with *pyrE* restored | This study |
| 922 | 630Δ*erm* Δ*IIDPQ*-p | 630Δ*erm*Δ *IID*Δ*IIP*Δ*IIQ* with *pyrE* restored | This study |
| 946 | 630Δ*erm* Δ*IIM*/*IIM* | 630Δ*erm*Δ*IIM* with *IIM* in the *pyrE* locus | This study |
| 1003 | 630Δ*erm*Δ*pyrE* Δ*sipL* | 630Δ*erm*Δ*pyrE* with *sipL* deleted | This study |
| 1010 | 630Δ*erm* Δ*sipL*-p | 630Δ*erm*Δ*pyrE* with *sipL* deleted | This study |
| 1013 | 630Δ*erm* Δ*sipL*/*sipL* | 630Δ*erm*Δ*sipL* with *sipL* in the *pyrE* locus | This study |
| 1018 | 630Δ*erm*Δ*pyrE* Δ*IIDPMQ* | 630Δ*erm*Δ*pyrE* with *spoIID*, *spoIIP*, *spoIIQ*, and *spoIIM* deleted | This study |
| 1054 | 630Δ*erm*Δ*pyrE* Δ*IIDP* | 630Δ*erm*Δ*pyrE* with *spoIID* and *spoIIP* deleted | This study |
| 1057 | 630Δ*erm*Δ*pyrE* Δ*IIDPM* | 630Δ*erm*Δ*pyrE* with *spoIID*, *spoIIP*, and *spoIIM* deleted | This study |
| 1102 | 630Δ*erm* Δ*IIDP*-p | 630Δ*erm*Δ*IID*Δ*IIP* with *pyrE* restored | This study |
| 1105 | 630Δ*erm* Δ*IIDPM*-p | 630Δ*erm*Δ*IID*Δ*IIP*Δ*IIM* with *pyrE* restored | This study |
| 1108 | 630Δ*erm* Δ*IIDPMQ*-p | 630Δ*erm*Δ*IID*Δ*IIP*Δ*IIM*Δ*IIQ* with *pyrE* restored | This study |
| 1111 | 630Δ*erm* Δ*IIM*-p | 630Δ*erm*Δ*IIM* with *pyrE* restored | This study |
| 1144 | 630Δ*erm*/*mCherry-IVA* | 630Δ*erm* with *mCherry-IVA* in the *pyrE* locus | (Ribis *et al.*, 2017) |
| 1155 | 630Δ*erm*Δ*pyrE sigK*$^{-}$ | 630Δ*erm*Δ*pyrE sigK::ermB* | This study |
| 1158 | 630Δ*erm*Δ*sipL*/*sipL-mCherry* | 630Δ*erm*Δ*sipL* with *sipL-mCherry* in the *pyrE* locus | This study |
| 1249 | 630Δ*erm* Δ*IIDQ*/*IIDQ* | 630Δ*erm*Δ*IID*Δ*IIQ* with *spoIID-spoIIQ* in the *pyrE* locus | This study |
| 1263 | 630Δ*erm*Δ*IIP*/*mCherry-IVA* | 630Δ*erm*Δ*IIP* with *mCherry-IVA* in the *pyrE* locus | This study |
| 1306 | 630Δ*erm*/*cotE-mCherry* | 630Δ*erm* with *cotE-mCherry* in the *pyrE* locus | This study |
| 1309 | 630Δ*erm*Δ*IIM*/*cotE-mCherry* | 630Δ*erm*Δ*IIM* with *cotE-mCherry* in the *pyrE* locus | This study |
| 1315 | 630Δ*erm*Δ*IIM*/*mCherry-IVA* | 630Δ*erm*Δ*IIM* with *mCherry-IVA* in the *pyrE* locus | This study |
| 1319 | 630Δ*erm*Δ*IIP*/*cotE-mCherry* | 630Δ*erm*Δ*IIP* with *cotE-mCherry* in the *pyrE* locus | This study |
| 1389 | 630Δ*erm sigK*$^{-}$-p | 630Δ*erm sigK::ermB* with *pyrE* locus restored | This study |
| 1444 | 630Δ*erm*Δ*pyrE sigG*$^{-}$ | 630Δ*erm*Δ*pyrE sigG::ermB* | This study |
| 1462 | 630Δ*erm* Δ*IID*/*IID*$_{E101A}$ | 630Δ*erm*Δ*IID* with *spoIID*$_{E101A}$ in the *pyrE* locus | This study |
| 1465 | 630Δ*erm* Δ*IIDQ*/*IID*$_{E101A}$ | 630Δ*erm*Δ*IID*Δ*IIQ* with *spoIID*$_{E101A}$ in the *pyrE* locus | This study |
| 1480 | 630Δ*erm*Δ*pyrE* Δ*IIP*Δ*sipL* | 630Δ*erm*Δ*pyrE* with *spoIIP* and *sipL* deleted | This study |
| 1482 | 630Δ*erm*Δ*pyrE* Δ*IIM*Δ*sipL* | 630Δ*erm*Δ*pyrE* with *spoIIM* and *sipL* deleted | This study |
| 1485 | 630Δ*erm*Δ*pyrE sigE*$^{-}$ | 630Δ*erm*Δ*pyrE sigE::ermB* | This study |
| 1488 | 630Δ*erm*Δ*pyrE sigF*$^{-}$ | 630Δ*erm*Δ*pyrE sigF::ermB* | This study |
| 1520 | 630Δ*erm*Δ*IIP*/*sipL-mCherry* | 630Δ*erm*Δ*IIP* with *sipL-mCherry* in the *pyrE* locus | This study |
| 1522 | 630Δ*erm*Δ*IIM*/*sipL-mCherry* | 630Δ*erm*Δ*IIM* with *sipL-mCherry* in the *pyrE* locus | This study |

| | | | |
|---|---|---|---|
| 1535 | 630Δ*erm* Δ*IIP*/*gpr-IIP*$_{E309A}$ | 630Δ*erm*Δ*IIP* with *gpr-spoIIP*$_{E309A}$ in the *pyrE* locus | This study |
| 1541 | 630Δ*erm sigF*$^{-}$-p | 630Δ*erm sigF::ermB* with *pyrE* locus restored | This study |
| 1544 | 630Δ*erm sigE*$^{-}$-p | 630Δ*erm sigE::ermB* with *pyrE* locus restored | This study |
| 1593 | 630Δ*erm sigG*$^{-}$-p | 630Δ*erm sigG::ermB* with *pyrE* locus restored | This study |
| 1643 | 630Δ*erm* Δ*IIQ*/*IIQ*$_{H120A}$ | 630Δ*erm*Δ*IIQ* with *spoIIQ*$_{H120A}$ in the *pyrE* locus | This study |
| 1649 | 630Δ*erm*Δ*pyrE IIP*$^{-}$ | 630Δ*erm*Δ*pyrE spoIIP::ermB* | This study |
| 1672 | 630Δ*erm IIP*$^{-}$-p | 630Δ*erm spoIIP::ermB* with *pyrE* locus restored | This study |
| 1674 | 630Δ*erm* Δ*IIDQ*/*IIQ*$_{H120A}$ | 630Δ*erm*Δ*IID*Δ*IIQ* with *spoIIQ*$_{H120A}$ in the *pyrE* locus | This study |
| 1721 | 630Δ*erm* Δ*IIDQ*/*IID* | 630Δ*erm*Δ*IID*Δ*IIQ* with *spoIID* in the *pyrE* locus | This study |
| 1724 | 630Δ*erm* Δ*IIDQ*/*IIQ* | 630Δ*erm*Δ*IID*Δ*IIQ* with *spoIIQ* in the *pyrE* locus | This study |
| 1749 | 630Δ*erm* Δ*IIP*/*IIP*$_{E309A}$ | 630Δ*erm*Δ*IIP* with *spoIIP*$_{E309A}$ in the *pyrE* locus | This study |
| 2059 | 630Δ*erm*Δ*pyrE* Δ*IIPQ* | 630Δ*erm*Δ*pyrE*Δ*IIQ* with *spoIIP* deleted | This study |
| 2065 | 630Δ*erm*Δ*IIPQ*-p | 630Δ*erm*Δ*IIPQ* with the *pyrE* locus restored | This study |
| 2068 | 630Δ*erm*Δ*IIPQ*/*IIP*$_{E309A}$ | 630Δ*erm*Δ*IIPQ* with *spoIIP*$_{E309A}$ in the *pyrE* locus | This study |

***E. coli* strains**

| | | | |
|---|---|---|---|
| 41 | DH5α | F– Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK$^{-}$, mK$^{+}$) *phoA supE44* λ– *thi-1 gyrA96 relA1* | D. Cameron |
| 531 | HB101/pRK24 | F- *mcrB mrr hsdS20*(rB$^{-}$mB$^{-}$) *recA13 leuB6 ara-13 proA2 lavYI galK2 xyl-6 mtl-1 rpsL20* carrying pRK24 | C. Ellermeier (Fimlaid *et al.*, 2013) |
| 659 | pJS107/*sigE* | pJS107-*sigE* targeting 119 bp in HB101/pRK24 | (Fimlaid *et al.*, 2013) |
| 681 | pJS107/*sigK* | pJS107-*sigK* targeting 265 bp in HB101/pRK24 | (Fimlaid *et al.*, 2013) |
| 735 | pJS107/*sigG* | pJS107-*sigG* targeting 546 bp in HB101/pRK24 | (Fimlaid *et al.*, 2013) |
| 782 | pJS107/*sigF* | pJS107-*sigF* targeting 459 bp in HB101/pRK24 | |
| 1289 | pJS107/*IIP* | | |
| 1366 | pET21a-*IID*$_{Δ27aa}$ | pET21a-*spoIID*$_{Δ27aa}$ (deletion of N-terminal 27 aa) in BL21(DE3) | This study |
| 1539 | pMTL-YN3 | pMTL-YN3 in DH5α | (Ng *et al.*, 2013) |
| 1576 | pDSW1728 | Tetracycline-inducible *mCherry* | (Ransom *et al.*, 2015) |
| 1630 | pMTL-YN3-Δ*IIP* | pMTL-YN3-Δ*spoIIP* in HB101/pRK24 | This study |
| 1641 | pMTL-YN3-Δ*IIQ* | pMTL-YN3-Δ*spoIIQ* in HB101/pRK24 | This study |
| 1642 | pMTL-YN3-Δ*IID* | pMTL-YN3-Δ*spoIID* in HB101/pRK24 | This study |
| 1662 | pMTL-YN1C | pMTL-YN1C in HB101/pRK24 | (Ng *et al.*, 2013) |
| 1670 | pMTL-YN3-Δ*IIDQ* | pMTL-YN3-Δ*spoIID-spoIIQ* in HB101/pRK24 | This study |
| 1675 | pMTL-YN3-Δ*IIM* | pMTL-YN3-Δ*spoIIM* in HB101/pRK24 | This study |
| 1676 | pMTL-YN1C-*IIQ* | pMTL-YN1C-*spoIIQ* in HB101/pRK24 | This study |
| 1677 | pMTL-YN1C-*IID* | pMTL-YN1C-*spoIID* in HB101/pRK24 | This study |
| 1704 | pMTL-YN3-*sipL* | pMTL-YN3-Δ*sipL* in HB101/pRK24 | |
| 1711 | pMTL-YN1C-*IIM* | pMTL-YN1C-*spoIIM* in HB101/pRK24 | This study |
| 1718 | pMTL-YN1C-*sipL* | pMTL-YN1C-*sipL* in HB101/pRK24 | This study |
| 1768 | pMTL-YN1C-*mCherry-IVA* | pMTL-YN1C-*mCherry spoIVA* in HB101/pRK24 | This study |
| 1777 | pMTL-YN1C-*sipL-mCherry* | pMTL-YN1C-*sipL-mCherry* in HB101/pRK24 | This study |
| 1789 | pMTL-YN1C-*IIDQ* | pMTL-YN1C-*spoIID-spoIIQ* in HB101/pRK24 | This study |
| 1812 | pMTL-YN1C-*cotE-mCherry* | pMTL-YN1C-*cotE-mCherry* in HB101/pRK24 | This study |
| 1845 | pET28a-*IIP*$_{Δ27aa}$ | pET21a-*spoIIP*$_{Δ27aa}$ (deletion of N-terminal 27 aa) in BL21(DE3) | This study |
| 1901 | pMTL-YN1C-*IID*$_{E101A}$ | pMTL-YN1C-*spoIID*$_{E101A}$ in HB101/pRK24 | This study |
| 1902 | pMTL-YN1C-*gpr-IIP*$_{E309A}$ | pMTL-YN1C-*gpr-spoIIP*$_{E309A}$ in HB101/pRK24 | This study |
| 1967 | pMTL-YN1C-*IIQ*$_{H120A}$ | pMTL-YN1C-*spoIIQ*$_{H120A}$ in HB101/pRK24 | This study |
| 1997 | pMTL-YN1C-*IIP*$_{E309A}$ | pMTL-YN1C-*spoIIP*$_{E309A}$ in HB101/pRK24 | This study |

**Supplementary Table S2. Primers used in this study.**

| Primer | Name | Sequence |
|---|---|---|
| 532 | 3' Universal EBS | CGAAATTAGAAACTTGCGTTCAGTAAAC |
| 1177 | 5’ NotI *spoIIQ* (*CD0125*) | AGAATGCGGCCGCCCACATTACTACACAGATAC |
| 1178 | 3' XhoI *spoIIQ* | AATACTCGAGGTTATACTATTACTTAATTAGACTC |
| 1487 | 5' IBS1 *spoIIP* 798 (*CD2469*) | AAAAAAGCTTATAATTATCCTTACTAAGCTGTTTTGTGCGCCCAGATAGGGTG |
| 1488 | 3' EBS1d *spoIIP* 798 | CAGATTGTACAAATGTGGTGATAACAGATAAGTCTGTTTTCTTAACTTACCTTTCTTTGT |
| 1489 | 5' EBS2 *spoIIP* 798 | TGAACGCAAGTTTCTAATTTCGATTCTTAGTCGATAGAGGAAAGTGTCT |
| 1558 | 5' *spoIID* (42) (*CD0126*) | GCTCTGTGTTAGTTCCATCAC |
| 1560 | 5' *spoIIP* (124) | GAAGCTAAAGTCGAAGGAAAT |
| 1561 | 3' XhoI *spoIIP* no stop (1018) | ATTACTCGAGATTTTTTTGTTTAAAATATTC |
| 1563 | 5' CD1221 (61) *spoIIM* | GGATTGTTGTTTATGATGTCTATT |
| 1565 | 3' CD1221 (532) *spoIIM* | CAACCCCAATGATAGTGCTATTA |
| 1572 | 5' NotI *spoIID* | AAAAGCGGCCGCGCTACTGATTTAAGAGCAGG |
| 1573 | 3' XhoI *spoIID* | ATTACTCGAGGATTATTGACGTGTTTTTGG |
| 1576 | 5' NotI *spoIIM* (*CD1221*) | AAAAGCGGCCGCCAACAGGTCTTACTCCAGG |
| 1577 | 3' XhoI *spoIIM* | ACAAGCTCGAGGATGCCACTGTATTCTCTG |
| 1631 | 5' NheI $His_6$-$SpoIID_{\Delta 27aa}$ | AAAGCTAGCATGCATCATCATCATCATCATAAAAATGTAGAATTAACTG |
| 1632 | 3' XhoI *spoIID* with stop | ACTCGAGTTAGTATATATCTTTTATTTTTG |
| 1658 | 3' *spoIID* (367) for qRT | CTACATATGTTCTAGCTGCTAC |
| 1759 | 5' Δ*sipL* SOE (*CD3567*) | GATGTAATTAAAGTTGACAATAGAATAGACCCTGGGAAGTGTCTTATTTTAG |
| 1760 | 3' Δ*sipL* rev oes | CTAAAATAAGACACTTCCCAGGGTCTATTCTATTGTCAACTTTAATTACATC |
| 1850 | 5' *spoIIQ* H120A SOE | TCTAAAACACTTGATGTTTGGGAAACTGCTAAAGGTGTAGATATTAGTTGTACTAAAG |
| 1851 | 3' *spoIIQ* H120A rev oes | CTTTAGTACAACTAATATCTACACCTTTAGCAGTTTCCCAAACATCAAGTGTTTTAGA |
| 1941 | 5' AscI Δ*spoIIP* | AATGGCGCGCCTTAGCATTTAATTTATACTGGAGGTTTATATG |
| 1942 | 5' Δ*spoIIP* SOE | GACTTTTATATTAGTATGCATTTTACCAGGAGCCGAGATTTTGGATGAATATTTTAAAC |
| 1943 | 3' Δ*spoIIP* rev eos | GTTTAAAATATTCATCCAAAATCTCGGCTCCTGGTAAAATGCATACTAATATAAAAGTC |
| 1944 | 3' SbfI Δ*spoIIP* | ATTCCTGCAGGTCCTCTGGTCTTGCACTTG |
| 1945 | 5' Δ*spoIIP* flanking | CTGACAAATCACTGACTAAACCC |
| 1946 | 3' Δ*spoIIP* flanking | CTTCATTATCTCCTTTAGGTGG |
| 1959 | 5' AscI Δ*spoIID* | TTAAGGCGCGCCGTGCAAAGATAGAAATGGACCATGG |
| 1960 | 5' Δ*spoIID* SOE | CCATTGGTTGTTTTGTTAGGATTTGTGGAGGGCTATAAATATTATGATATATTATCAC |
| 1961 | 3' Δ*spoIID* rev eos | GTGATAATATATCATAATATTTATAGCCCTCCACAAATCCTAACAAAACAACCAATGG |
| 1962 | 3' SbfI Δ*spoIID* | CTACCTGCAGGCGGTTTGTCTTACTGTAGTG |
| 1963 | 5' Δ*spoIID* flanking | GCCAGGAGGATGTGCTATAG |
| 1964 | 3' Δ*spoIID* flanking | CCTTCTCATATTTTAATTTCGTTGCTAATCC |
| 1965 | 5' AscI Δ*spoIIQ* | TTAGGCGCGCCCAGAGAGCCCAGTATCTATAAAC |
| 1967 | 5' Δ*spoIIQ* SOE | GTTTGTTTATTAGCAGTAGGTGGAGTTTGGGGTATACATGTGCATTTAGAGGCG |
| 1968 | 3' Δ*spoIIQ* rev eos | CGCCTCTAAATGCACATGTATACCCCAAACTCCACCTACTGCTAATAAACAAAC |
| 1969 | 3' SbfI Δ*spoIIQ* | TTGCCTGCAGGCTATGGCAGCTGCTATTGGTTC |
| 1970 | 5' Δ*spoIIQ* flanking | GGTGGTCAAATCGAAATAGTAGAAG |
| 1971 | 3' Δ*spoIIQ* flanking | CTCTGCTGTTCTTTCTCCTATAAG |
| 1973 | 5' Δ*spoIIDQ* SOE | GGATTTGTGACTTGCTCTGTGGCGTATAGTGGAGAAAAATCTATAGAC |
| 1974 | 3' Δ*spoIIDQ* rev eos | GTCTATAGATTTTTCTCCACTATACGCCACAGAGCAAGTCACAAATCC |
| 1995 | 5' AscI Δ*sipL* Gibson | gtcaattgttcaaaaaaataatggcGGCGCGCCGCTCAACTTGGAATACCTGC |
| 1997 | 5' Δ*spoIIM* flanking | GATGCAGTATTTGTCAGTATG |
| 1998 | 3' Δ*spoIIM* flanking | CTATACCATCTATGTTTC |
| 1999 | 5' AscI Δ*spoIIM* | gtcaattgttcaaaaaaataatggcGGCGCGCCGATTGAACAAGGCTCTATTC |
| 2000 | 5' Δ*spoIIM* SOE | agcaaggcaagaccgatcgggcccCCTGCAGGGTACTTACATCCTTACTATCAC |

| | | |
|---|---|---|
| 2001 | 3' Δ*spoIIM* rev eos | TAGACAAATAATTATTATAGGATTGGCAAGTATTGGTATTATTAAGTTCCTAG |
| 2002 | 3' SbfI Δ*spoIIM* | CTAGGAACTTAATAATACCAATACTTGCCAATCCTATAATAATTATTTGTCTA |
| 2088 | 3' SbfI Δ*sipL* gibson | agcaaggcaagaccgatcgggccccctgcaggGTTTGTTAGCTTTTCTCCAATACCTTG |
| 2133 | 3' XhoI *mCherry* Gibson | gccaagcttgcatgtctgcaggcctcgagTTATTTATATAATTCATCCATACCTCCTGTTG |
| 2138 | 3' *spoIIM* internal colony PCR | GCAACCCCAATGATAGTGC |
| 2165 | 5' NotI *sipL* Gibson | ggaattagggatgtaataagcggccgcGAGAGACATATAGGAAAGAAAATATTG |
| 2195 | 3' *spoIID* internal to deletion | GACCTTTAGTTCCTCTAACTGCTTC |
| 2196 | 3' *spoIIQ* internal to deletion | CACCTTTATGAGTTTCCCAAACATC |
| 2197 | 3' *spoIIP* internal to deletion | CCATCCTTTGCTATCTAAAGCACTAG |
| 2211 | 5' SipL-mCherry linker SOE | CTTATTTTAGAAAAAAAAGTCGTATTAGTAGATGCAATGGTATCTAAAGGAGAAGAAGAT |
| 2212 | 3' SipL-mCherry linker rev | ATCTTCTTCTCCTTTAGATACCATTGCATCTACTAATACGACTTTTTTTTCTAAAATAAG |
| 2231 | 5' NotI *spoIID* Gibson | ggaattagggatgtaataagcggccgcCGCTACTGATTTAAGAGCAGG |
| 2232 | 3' XhoI *spoIIQ* Gibson | caagcttgcatgtctgcaggcctcgagGTTATACTATTACTTAATTAGACTC |
| 2263 | 5' CotE-mCherry SOE (*CD1433*) | CTTGAAGGTATTTATGGGCAATTCGCAATGGTATCTAAAGGAGAAGAAGAT |
| 2264 | 3' CotE-mCherry rev eos | ATCTTCTTCTCCTTTAGATACCATTGCGAATTGCCCATAAATACCTTCAAG |
| 2268 | 5' NotI *cotE* Gibson | ggaattagggatgtaataagcggccgcTTCAATCCACTCACCTCGTATAATC |
| 2297 | 5' NcoI *spoIIP* pET28a Gibson | GTTTAACTTTAAGAAGGAGATATACCATGGCACAAGATGATTTTTTAAAGTTTTTAG |
| 2316 | 3' XhoI *spoIIP* pET28a Gibson | CAGTGGTGGTGGTGGTGGTGCTCGAGATTTTTTTGTTTAAAATATTCATCCAAAATCTC |
| 2352 | 5' NotI *gpr* Gibson | ggaattagggatgtaataagcggccgcCCTATATAAATAGTTGCTACCTTTTTCTG |
| 2378 | 3' XhoI *spoIID* YN1C Gibson | caagcttgcatgtctgcaggcctcgagGATTATTGACGTGTTTTTGGCATTTTATAC |
| 2379 | 5' *spoIID* E101A SOE | CTATTTATGTGGAGTACTAGCTGGTGCGATGTCTTCAGAGTTTGATATAGAAGC |
| 2380 | 3' *spoIID* E101A rev eos | GCTTCTATATCAAACTCTGAAGACATCGCACCAGCTAGTACTCCACATAAATAG |
| 2381 | 5' *spoIIP* E309A SOE | GTTTGCAGTTAAGAATCATATGTTAGTAGCAATAGGGAATAACGCAACAAGTGTAG |
| 2382 | 3' *spoIIP* E309 rev eos | CTACACTTGTTGCGTTATTCCCTATTGCTACTAACATATGATTCTTAACTGCAAAC |
| 2383 | 3' XhoI *CD2468* YN1C Gibson | caagcttgcatgtctgcaggcctcgagCTACTTATTAAATTTTTTTATGATTTCATG |
| 2449 | 5' NotI *spoIIQ* Gibson | ggaattagggatgtaataagcggccgcCACATTACTACACAGATACAAAAATAAAAG |
| 2481 | 5' NotI *spoIIP* 269 gibson | ggaattagggatgtaataagcggccgcACTCTTACAATAGATGTCTTAGATATGGC |

Restriction sites are underlined.

**Supplementary Table S3 -** Sporulation heat resistance ratios determined for all strains across all replicates.

This table can be accessed using the following link:

**https://www.dropbox.com/s/ew6lylqvovbl6qp/Supplementary%20Table%20S3.xlsx?dl=0**

Heat resistance (H.R.) efficiencies were determined from 23 hr cultures and represent the mean and standard deviation for a given strain relative to wild type based on a minimum of three independent biological replicates. The limit of detection of the assay is $10^{-6}$.

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