Characterisation of the *Clostridium difficile* SpoIIDMP complex and its role in forespore engulfment

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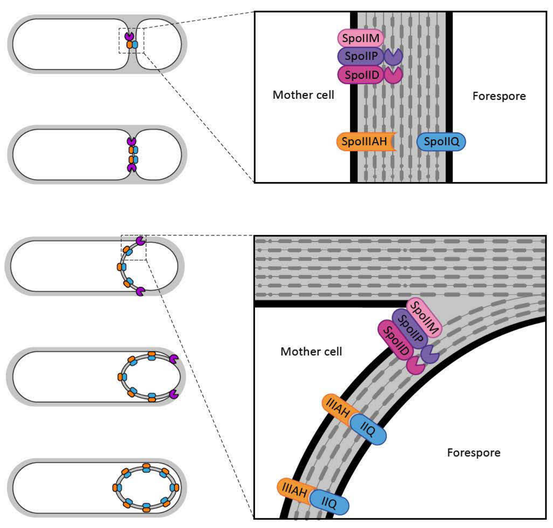
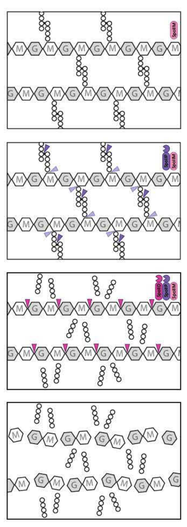
/// Background

Clostridium difficile is the most common cause of antibiotic-associated intestinal infections in humans and a significant cause of morbidity and mortality worldwide. Spores are thought to be the primary infective agent and represent an attractive target for intervention. A detailed understanding of the essential cellular processes that underpin *C. difficile* sporulation could thus aid in the development of effective therapeutics.

One of the critical stages of sporulation is engulfment, during which the mother cell membrane envelops the newly formed forespore to form a 'cell-within-a-cell' structure. In *B. subtilis*, this process is facilitated by a protein complex consisting of cell wall remodelling enzymes: SpoIID and SpoIIP, as well as SpoIIM, which is thought to play a structural role within the complex. These act on cell wall peptidoglycan in a sequential, complementary and costimulatory manner pulling the mother cell membrane around the forespore and have all been shown to be essential in spore formation.



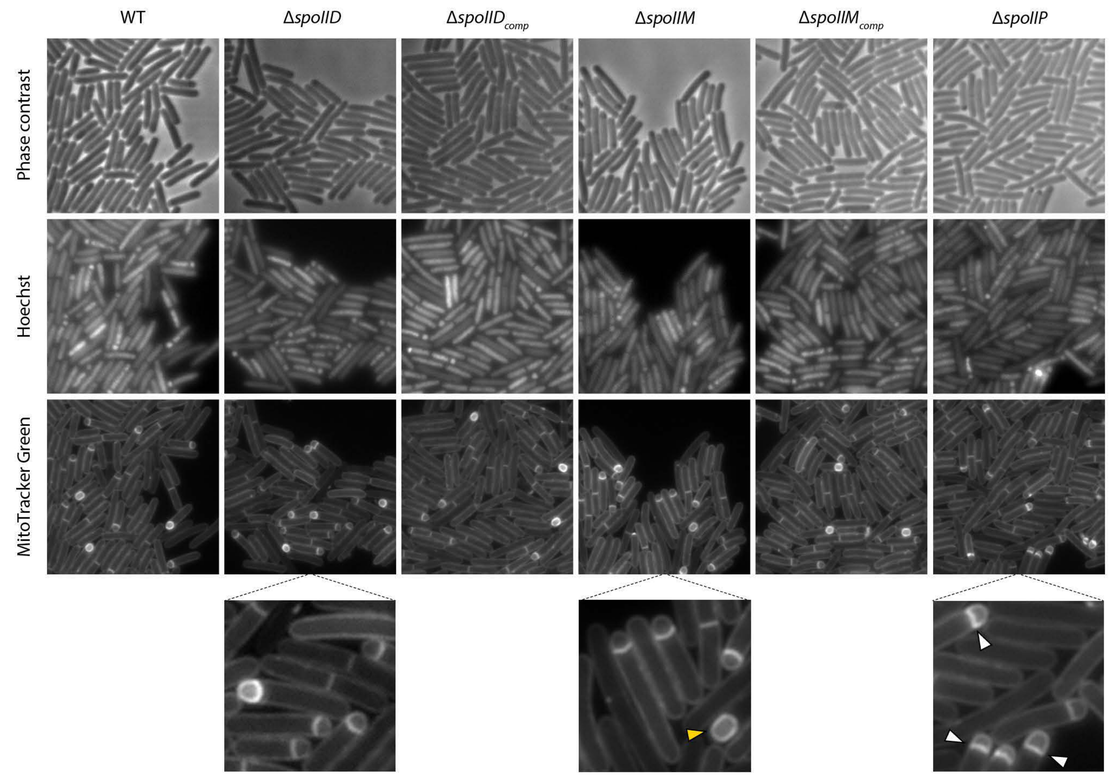


SpoIIM
- localizes to the septum and acts as a scaffold for the complex
- recruits SpoIIP

SpoIIP
- amidase activity (removes stem peptides)
- D,D-endopeptidase activity (cleaves peptide cross-links)
- recruits SpoIID

SpoIID
- lytic transglucosylase (cleaves denuded glycan chains)
- stimulates SpoIIP activity

/// Spore morphogenesis



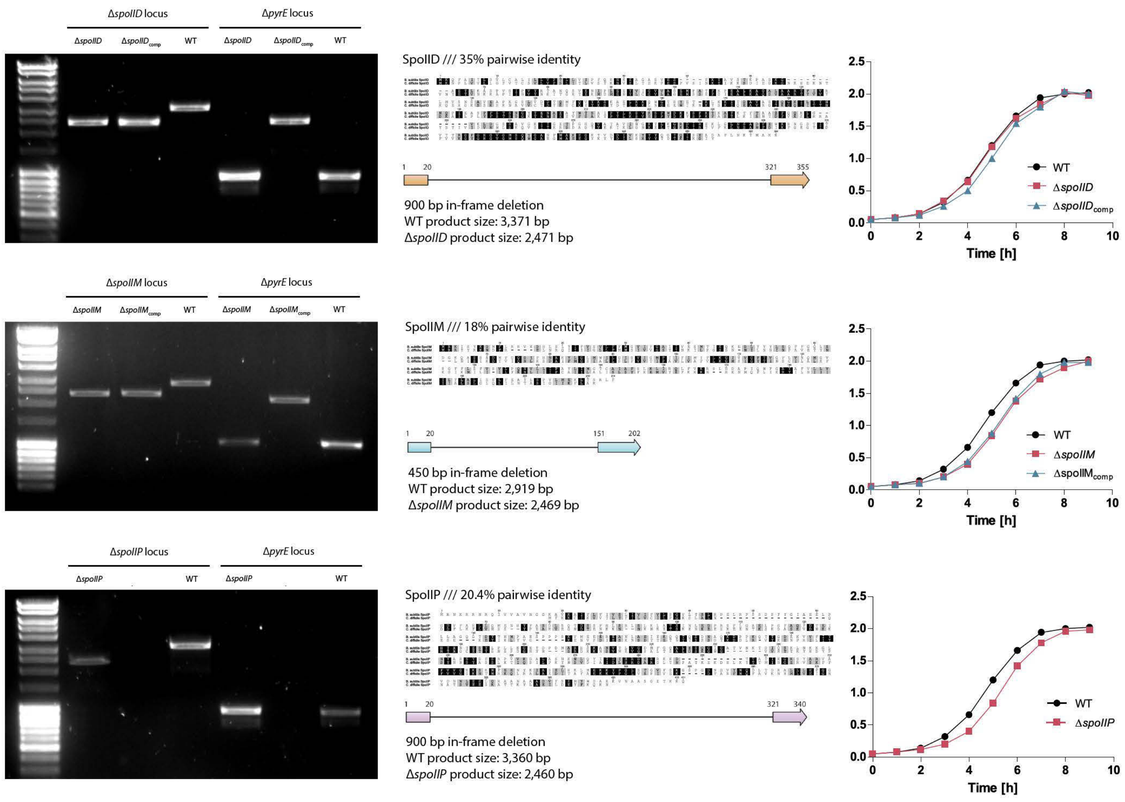
To identify which stage of the sporulation process is affected by disrupting *spoIIDMP* expression, 14h cultures grown in SM broth were stained with Hoechst and MitoTracker Green and analysed by phase contrast and fluorescence microscopy. Both the *spoIID* and the *spoIIP* mutant were blocked at the engulfment stage of sporulation as indicatd by the absence of fully formed sporangia. This phenotype was more severe in the *spoIIP* mutant in which inverted septa could be observed (white arrows), indicative of forespore collapse. This is consistent with SpoIIP being necessary for SpoIID recruitment as observed in *B. subtilis*. As seen previously, disruption of *spoIIM* expression did not prevent spore formation, although the number of fully engulfed forespores (yellow arrows) was significantly lower than that seen in WT.

/// Construction of *spoIIDMP* mutants in *C. difficile*

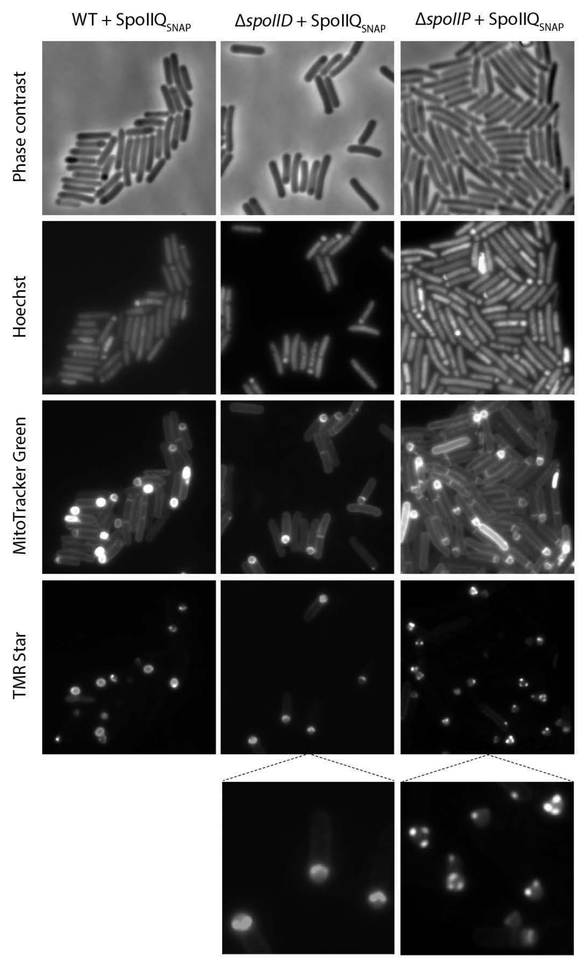
The *C. difficile* genome encodes three distant orthologues of SpoIID, SpoIIM and SpoIIP. Allele-coupled exchange (ACE) was used to create in-frame deletions in the corresponding genes in *C. difficile* 630Δ*erm*. The resulting mutants were then complemented by re-introducing the WT gene under its native promoter immediately downstream of *pyrE*.

In order to assess the effect of disrupting *spoIIDMP* expression, growth was monitiored in nutrient medium over a period of 9h. No significant growth defect was observed for any of the mutants, consistant with their dispensible role during vegetative growth.

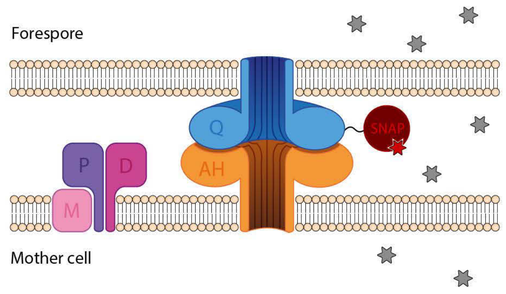
** Note:* the *spoIIP* mutant could not be complemented despite multiple attempts at subcloning the WT gene under its native promoter, possibly due to toxicity in *E. coli*. To address this issue, the gene will be cloned under the control of an inducible Ptet promoter.



/// SpoIIQ localization in *spoIID* and *spoIIP* mutants

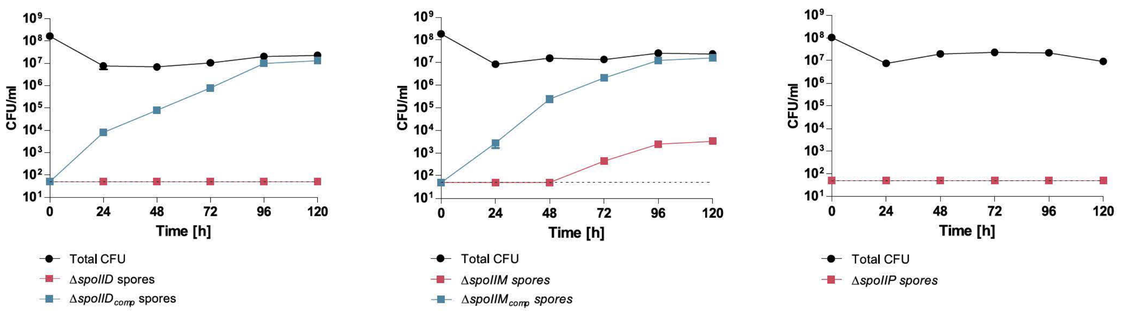


The forespore-expressed SpoIIQ preotein interacts with SpoIIIAH produced in the mother cell to form a putative channel linking the two compartments and acting as a redundant mechanisms of engulfment. In *B. subtilis*, SpoIIQ is localized to the septal membrane in a SpoIIIAH and a SpoIIDMP-dependant manner. To test whether disruption of the SpoIIDMP complex components affects SpoIIQ localization, the *spoIID* and the *spoIIP* mutants were episomally complemeted with a SNAP-tagged copy of SpoIIQ. While SpoIIQ localization was not affected in the *spoIID* mutant, the *spoIIP* mutant showed a disctinct punctate staining with clear foci at the leading edge of the engulfing membrane suggesting that SpoIIP might be required for the septal localization of SpoIIQ.



/// Sporulation dynamics

To asssess the impact of *spoIIDMP* disruption on sporulation, sporulation dynamics in liquid medium were monitored over a 5 day period by measuring heat resistant CFU formation at 24h intervals. Both the *spoIID* and the *spoIIP* mutant showed a severe sporulation defect and failed to produce any heat reistant spores. While the *spoIIM* mutant did form spores, sporulation was delayed and its endpoint efficiency was 2-log lower than that observed for WT. This is consistent with results obtained through a forward genetic study of gene essentiality (Dembek et al, 2015) in which *spoIID* and *spoIIP* but not *spoIIM* were identified as required for sporulation. For both the *spoIID* and the *spoIIM* mutant, re-introduction of the WT gene under its native promoter onto the chromosome restored the WT phenotype.



/// Conclusions

- The SpoIIDMP complex is dispensible for normal growth under the conditions tested
- SpoIID and SpoIIP are required for efficient sporulation in *C. difficile* as their disruption results in a block at the early stage of the engulfment process
- In contrast to what has been observed in *B. subtilis*, disruption of SpoIIM significantly reduces sporulation efficency but does not prevent spore formation
- SpoIIP may be involved in localizing SpoIIQ to the septal membrane

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