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TITLE: MURAMIC-Δ-LACTAMS ARE INVOLVED IN *C. difficile* SPORULATION, GERMINATION AND VIRULENCE

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Background and aims
Spores are produced by many organisms as the result of a survival mechanism, triggered under several environmental conditions. They are multi-layered structures, one of which is a peptidoglycan layer known as the cortex. The cortex peptidoglycan has been described for several organisms, including *B. subtilis* and *C. perfringens*, but has yet to be published for *C. difficile*. Compared to the vegetative cell peptidoglycan, the cortex peptidoglycan possesses a unique, modified sugar called muramic-δ-lactam, synthesized by at least two enzymes: an amidase CwlD and an N-deacetylase PdaA. In this work, we analyzed the *C. difficile* cortex structure, we characterized the N-deacetylase involved in muramic-δ-lactam synthesis and investigated the impact of muramic-δ-lactams on *C. difficile* physiology and virulence.

Methods
The cortex of *C. difficile* 630Δ*erm* and *pdaA* mutant strains were analyzed using UHPLC coupled HRMS. Germination was assessed through optical density monitoring of spore suspensions after addition of taurocholate. Spore resistance properties were investigated by enumeration of spore suspensions after treatment with ethanol, hydrogen peroxide or heat. Sporulation was studied in liquid cultures after 72H of growth. Morphology of both strains was assessed through transmission electron microscopy.

Results
The cortex analysis revealed several differences between the *B. subtilis* and *C. difficile* cortex structures. For instance, only 24% of muropeptides in *C. difficile* carried muramic-δ-lactams, compared to 50% of muropeptides in *B. subtilis*. Analysis of the cortex from the *pdaA* mutant showed minor traces of muramic-δ-lactams (0.4% of all muropeptides). Investigation of the consequences of this decrease in muramic-δ-lactams in the *pdaA* mutant showed a decreased sporulation rate, an altered germination, and a decreased heat-resistance. In a virulence assay, the *pdaA* mutant also showed a delayed virulence.

Conclusions
Surprisingly, our results suggest a much broader impact for muramic-δ-lactams in *C. difficile* compared to previously characterized model organisms, such as *B. subtilis*. Our results highlight a novel factor linking both the germination and sporulation processes, and provide an insight into a new strategy to target *C. difficile* and its dissemination by targeting enzymes involved in cortex synthesis.