1. INTRODUCTION

Bacillus spores are dormant cells that exhibit high resistance to environmental stresses [1]. Spores consist of multiple concentric shells encasing dehydrated genetic material at the centre (the core). One of these shells is a loosely cross-linked peptidoglycan layer, called the cortex, surrounding the core. Encasing this is the coat, which exhibits a unique folding geometry (figure 1a–c). The coat protects the genetic material while permitting the diffusion of water and small molecules to the spore interior. Paradoxically, the coat must be chemically resilient and physically tough [5–7] but still possess significant mechanical flexibility [4,8,9]. During germination, the coat must be broken apart so that it can be rapidly shed [10].

The ridges of the Bacillus subtilis coat emerge during the process of sporulation in which water is expelled from the spore core and cross links occur in the cortex [5,10–14]. The mature spore is not static. It expands and contracts in response to changes in relative humidity [4,8,9]. Although ridges are present in spores of many if not most species [2,15–17] of the family Bacillaceae, these ridges are very poorly understood; we do not understand the forces guiding their formation, how their topography is influenced by the coat’s material properties or their biological function, if any.

To address these questions, we first considered that ridges could emerge spontaneously, as in the case of wrinkles that form when a thin layer of material that adheres weakly to a support is under compression [18]. The coat and cortex form such a system, because the core volume (and, therefore, its surface area) decreases during sporulation [19]. Rucks can form if the stress in the system overcomes the adhesive forces between the coat and the underlying cortex [20]. Consequently, we investigated the role of mechanical instabilities in the formation of ridges and the implications of this mechanism for spore persistence.

2. RESULTS

The height profiles of a B. subtilis in figure 1d show that a partial unfolding of the ridges accompanies the expansion of the spore at high humidity. We obtained similar results for Bacillus anthracis spores (figure 1c). Furthermore, fully hydrated Bacillus atrophaeus spores were previously shown to exhibit similar characteristics [4], indicating that this behaviour is not limited to a single species and raising the possibility that it is ubiquitous.

To analyse whether ruck formation can explain the characteristic wrinkle patterns and their response to increased relative humidity, we modelled the coat and cortex as two adhered concentric rings and calculated the response of this structure to gradual reductions in volume, using a combination of scaling analyses and numerical simulations (see the electronic supplementary material). We restricted our model to two dimensions, both because the wrinkle morphology is that of long ridges along the spore, and in order to focus on a minimal model. Typical parameter values in our model are as follows: core radius $R_{\text{core}}$, and thickness, $h$, of the coat to be approximately 300 and 40 nm, respectively [2,21,22] (see the electronic supplementary material, table S1).
the measured elastic modulus of the *B. subtilis* coat $E$ approximately 13.6 GPa using an atomic force microscope [23] (AFM), and the energy of adhesion between the coat and the cortex, $J$, approximately 10 J m$^{-2}$, associated with non-specific electrostatic interactions between the cortex peptidoglycan [24] and the coat [24,25]. We note that the presence of outer forespore membrane can affect the strength of adhesion between the coat and cortex; however, we assumed that this membrane is no longer present in the mature spore.

The simulations show that as the rings shrink when the strain is larger than a critical threshold, $c$, the coat first buckles to form a symmetric wavy pattern around the cortex. This pattern then loses stability to delamination to form rucks (figure 2a–c). Once the rucks are formed, regaining spore volume does not result in reattachment of the coat. Rather, rucks unfold by decreasing their height and increasing their width (figure 2d), in qualitative agreement with our biological observations in figure 1d.

### 3. DISCUSSION

Wrinkles formed according to the mechanism in figure 2a–c are persistent. They do not readily attach back to the cortex, because they arise owing to a subcritical (nonlinear) instability. This has implications for the dormant spore, because it suggests that after completion of sporulation the spore volume can increase or decrease in response to ambient relative humidity without a significant resistance from the coat. The persistence of rucks ensures that the coat remains in a flexible state, despite large changes in the volume during dormancy [4,8,9], thereby providing a mechanism for maintaining structural integrity of the spore.

The origin of the coat’s flexibility in the wrinkled state can be best understood by comparing the energy cost of bending and compression. If the coat were fully attached to the cortex, shrinkage of the spore interior owing to dehydration would have required the coat to be compressed. In contrast, our results indicate that the wrinkled coat expands and shrinks by changing its local curvature. While the energy cost of coat compression scales linearly with the coat thickness $U_{\text{compression}} \sim h$, the energy cost of bending scales with the third power of thickness $U_{\text{bending}} \sim h^3$. This means that as a layer of material gets thinner, bending becomes easier relative to compression. In the case of spores, reductions in the internal volume of the spore are best accommodated by folding and unfolding of wrinkles.

Figure 1. *B. subtilis* spore morphology. (a) Wild-type (strain PY79) spores were analysed by SEM, (b,c) TEM [2] (by fixation in glutaraldehyde and osmium, dehydration, embedment in Spurr’s resin, and then thin-section transmission electron microscopy) and (d) with AFM height profiles. Separate height profiles obtained on *sterne* strain of *B. anthracis* are also given in (e). (c) Is a magnification part of (b). Cortex (Cx), inner coat (IC), outer coat (OC) and a ruck (R) are indicated in (b) and/or (c). The size bars indicate (a) 250, (b) 500 and (c) 100 nm. (The outer most layer of the coat, the crust [3] is not seen because of the fixation method). The spore has an ovoid shape with a long and a short axis [4]. (d) Height profiles measured along the short axis of a spore are recorded at low (35%, solid line) and high (95%, dashed line) relative humidity depict partial unfolding of the wrinkles at a high relative humidity. (e) Height profiles measured for *B. anthracis sterne* also exhibit partial unfolding of the wrinkles at a high relative humidity. To plot the two curves as closely as possible, an offset is added to the height profiles at a low relative humidity because the overall height of the spore increases with relative humidity. Note that the widths of the spores are larger than the widths of the curves in (d,e), which are plotted across the ridges on top of the spore.
According to the wrinkle model, if the spore internal volume continues to increase, the wrinkles will eventually unfold completely. Beyond that point, the coat will begin to resist any further increase in volume. The biological observation in figure 1 that the wrinkles do not unfold completely suggests that this point is not reached even at very high relative humidity or at full hydration. Therefore, expansion of the dormant spore is not limited by the coat. Instead, the cortex of the dormant spore must have a limited ability to swell. Consistent with this view, \textit{B. subtilis} spores lacking most of the coat owing to mutations in \textit{cotE} and \textit{gerE} \cite{27} were not larger than wild-type spores at a high relative humidity (figure 3). This particular mutant no longer has the resistance properties of wild-type spores, especially to lysozyme; however, they maintain viability in laboratory environment \cite{12,28}. The cortex’s limited ability to swell can be explained by its rigidity, as our AFM-based mechanical measurements \cite{23} on the \textit{cotE gerE} mutant revealed an elastic modulus around approximately 6.9 GPa. A rigid cortex is also needed to sustain pulling forces on the coat, as well as in creating a tight girdle around the dehydrated core.

The observed architecture and dynamics of the coat and cortex of the dormant spore have important implications for the role of mechanical processes involved in germination, as well. In contrast to the dormant state, the wrinkles disappear in germinating spores \cite{17,19}. Considering our model of wrinkle formation in the coat, a loss of cortex’s ability to sustain the pulling forces exerted on the coat can lead to coat unfolding. Degradation of the cortex peptidoglycan during germination can plausibly facilitate this process. In fact, mutant spores that lack the capacity for the degradation of their cortex peptidoglycan still maintain wrinkles in their coats even after triggering germination and partial hydration of their core \cite{29}. We note that according to this mechanism, the unfolding of the coat during germination is not necessarily driven by the expansion of the spore interior, but to a significant degree by the relaxation of the coat to its unfolded state. Because the unfolded and relaxed coat has a larger volume, relaxation of the coat could act like

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\textbf{Figure 2.} Model of formation of folds in the spore coat and their response to spore shrinking. (\textit{a}–\textit{c}) Simulation of the ruck formation as the radius of spore interior (\textit{Rin}) shrinks during sporulation. \textit{Rin} values are given as percentages of the initial value, 300 nm. \textit{Rout} is the average outer diameter. Using bending and stretching modulus values estimated from thickness and mechanical measurements of the coat, the model predicts the emergence of rucks that are comparable in width, height and number to previous reports \cite{26}. (\textit{d}) Upon spore expansion, rucks formed during sporulation do not reattach readily, but rather decrease their height and increase their width. Details of simulation results are given in electronic supplementary material, movie S1. (Online version in colour.)
a pump, driving water into the spore. While hydration forces are likely to be primarily responsible for core hydration, a relaxed coat would allow core to absorb a larger volume of water.

Our findings raise the possibility that the mechanical properties of the coat participate in coat shedding, a prerequisite to outgrowth. The *B. subtilis* coat is shed as two hemispheres during or immediately following germination [19]. Possibly, the coat’s mechanical properties play a role in holding these hemispheres together prior to germination, or facilitate their separation prior to the first cell division after germination.

4. CONCLUSION

Our findings suggest that the coat’s global mechanical properties are critical not only during dormancy but also, strikingly, for rapidly breaking dormancy upon germination. We propose that the coat takes advantage of mechanical instabilities to fold into a wrinkled pattern during sporulation and accommodate changes in spore volume without compromising structural and biochemical integrity. Importantly, we argue that the emergent properties of the assembled coat, such as its elastic modulus and thickness [17], rather than specific individual molecular components, are responsible for coat flexibility. In this view, a functional coat can be built in a large number of ways and with diverse protein components. Such freedom in design parameters could facilitate evolutionary adaptation (particularly with respect to material properties) and the emergence of the wide range of molecular compositions and arrangements found among *Bacillus* spore coats [2,30]. The spore and its protective coat represent a simple paradigm likely used in diverse cell types [31] where regulated flexibility of a surface layer is adaptive, and may inspire novel applications for a controlled release of materials.

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