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14. ABSTRACT The bacterial endospore is a uniquely resistant and highly differentiated form of bacterial cell. Its unique structure confers resistance to a variety of conditions such as heat, pressure, radiation, dehydration and chemicals. Nevertheless, it is able to respond rapidly to an appropriate chemical stimulus, undergoing biophysical and degradative changes that remove the specialised layers of coat and cortex, and restore the activities of a vegetative cell. Two major proteases of broad specificity have been detected in spore coat layers. One, AprX, is released into the supernatant during germination, both in a free form and associated with high molecular weight complexes. The second, a 30kDa protease, is retained in the spore. Mutant spores lacking AprX overproduce the 30kDa protease, possibly in a compensatory manner. These proteases of broad specificity are likely to be involved in the breakdown of spore coat proteins during germination. A strain with an unusually alkali-resistant spore coat was identified as <i>Brevibacillus borstelensis</i>. Coat proteins were successfully extracted by boiling at neutral pH with high concentrations of reducing agent and detergent. This strain germinated very slowly and asynchronously, making it impractical to undertake biochemical studies of spore germination or coat degradation.					
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Resistant Bacterial Spore Coats and Their Breakdown during Germination

(Final report for EOARD)

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Principal Investigator: Prof Anne Moir

Professor of Microbiology
Dept. of Molecular Biology & Biotechnology
University of Sheffield
a.moir@sheffield.ac.uk

Secondary Investigator: Dr Raymond Allan

Post-doctoral Researcher
Dept. of Molecular Biology & Biotechnology
University of Sheffield
r.allan@sheffield.ac.uk

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Summary

The bacterial endospore is a uniquely resistant and highly differentiated form of bacterial cell. Its unique structure confers resistance to a variety of conditions such as heat, pressure, radiation, dehydration and chemicals. Nevertheless, it is able to respond rapidly to an appropriate chemical stimulus, undergoing biophysical and degradative changes that remove the specialised layers of coat and cortex, and restore the activities of a vegetative cell.

This project has aimed to understand how the spore coat is modified during germination, and how such modification is related to other germination-associated events in the spore. The project has established:

1. Only a small proportion of the total spore coat protein complement is released during *Bacillus subtilis* germination. It appears that most of the spore coat proteins are degraded *in situ*, as the amount of protein released into the supernatant was relatively low (<1% of total spore protein). Nevertheless, five proteins have been identified as major components in the complex profile of released protein material. Of these, only one is a bona fide coat protein, GerQ (YwdL).
2. Of the four others identified, YhcN is implicated in germination and outgrowth, and Ndk, a nucleotide diphosphate kinase, in spore formation (Misra et al, 2009). Roles of YlbF and YlaK remain to be elucidated.
3. In circumstances where the spore cortex is not hydrolysed, spore germination is initiated, and some degradative changes occur in spore coat layers. A number of coat associated proteins are released, notably CotA, CotQ, SodA, which are coat-associated enzymes, and YkuS, a protein of unknown function but specific to and conserved in endospore formers. Surprisingly, spore-specific SASP proteins are released into the outer layers of the spore (SspA, B) or released into the supernatant (SspE).
4. Two major proteases of broad specificity have been detected in spore coat layers. One, AprX, is released into the supernatant during germination, both in a free form and associated with high molecular weight complexes. The second, a 30kDa protease, is retained in the spore. Mutant spores lacking AprX overproduce the 30kDa protease, possibly in a compensatory manner. These proteases of broad specificity are likely to be involved in the breakdown of spore coat proteins during germination.
5. A strain with an unusually alkali-resistant spore coat was identified as *Brevibacillus borstelensis*. Coat proteins were successfully extracted by boiling at neutral pH with high concentrations of reducing agent and detergent. This strain germinated very slowly and asynchronously, making it impractical to undertake biochemical studies of spore germination or coat degradation.

Publication in preparation Subject to EOARD's approval for publication, paper(s) are in preparation from this work, on the protein release during germination, and the spore-associated proteases.

Results & Discussion:

Proteins released from the spore during germination.

As discussed in the July 2009 report, several proteins that are released from the spore coat of wild type spores during germination have been identified. Only one of these proteins (GerQ/YwdL), is a recognised coat protein (Ragkousi& Setlow, 2004). Others include Ndk, nucleoside diphosphate kinase, important in the interconversion of nucleoside triphosphates and formation of mature spores(Misra et al, 2009), YhcN (possible role in spore germination/outgrowth), YlbF (Tortosa et al., 2000; a potential Zn^{2+} binding protein), and YlaK, a predicted ATPase. **In supernatants of germinating *cwID* mutant spores (below) , these proteins released from wild type germinating spores are not predominant, suggesting that their release is dependent on cortex hydrolysis.**

Proteins released from *cwID* mutant spores.

During spore germination, spore cortex peptidoglycan is hydrolysed, and consequently the spore core rehydrates, allowing enzyme activities in the spore core to resume. These processes do not occur in a *cwID* mutant, although spore coat breakdown is initiated, and some proteins are released (Fig. 1). **This indicates that some coat protein changes can occur independently of cortex breakdown, and that cortex hydrolysis and full rehydration of the core are not needed for signal transduction from the germination apparatus to initiate changes in the spore coat.**

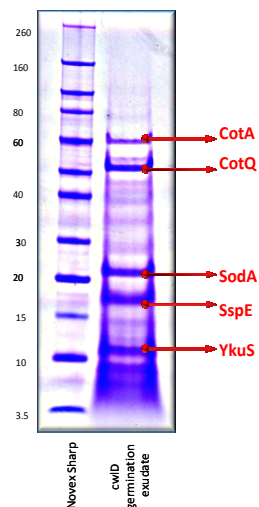


Fig. 1. Proteins released into the supernatant by germinating spores of a *B. subtilis cwID* mutant. Germination exudate (after 3 h germination in Tris-HCl [pH 8.0 / 100 mM], 50 mM KCl, 100 mM L-alanine) was filtered through Millex GP 0.22 μ m filters (Millipore, U.S.A) then concentrated in Vivaspin 20 columns (Sartorius, Germany) prior to separation on a Bis-Tris (4-12%) gel. After blotting onto Polyvinylidene Fluoride (PVDF) in NuPAGE transfer buffer, N-terminal sequencing was performed on highlighted bands. Molecular weight standards are in kDa.

The major proteins released into the supernatant in the *cwID* mutant instead represent a different subset of proteins, mainly spore-coat associated, some with enzyme activities.

These include CotA, an abundant outer spore coat protein (Imamura et al., 2010), which is a laccase (Martins et al, 2002); CotQ (YvdP) a likely FAD-linked oxidase reported in spore coats (Lai et al., 2003), SodA, a Mn-dependent superoxide dismutase implicated in spore coat assembly (Henriques et al., 1998) , and YkuS, a small (83aa) protein of unknown function, that is conserved in the endospore-forming Bacilli and Clostridia.

Finally, and unexpectedly, SspE, the major gamma-type SASP (small acid soluble protein) present in the inner cellular compartment of dormant spores, and normally hydrolysed during germination, is released into the supernatant of germinating *cwID* spores as a 17kDa form, possibly as a dimer.

Release of SASP proteins in a *cwID* mutant

In addition to the released SspE, other SASP proteins have been detected in an unusual state in partly-germinated *cwID* mutant spores. Proteins retained in the spore outer layers of germinated *cwID* mutant spores include alpha and beta SASPs, in complete and partially degraded forms (Fig 2). , N-terminal sequencing identified full length SspA (major component) and SspB (minor component) small acid-soluble proteins (SASPs) at 11kDa, and a 7 kDa version, processed to give N-termini at residues 11 and 13, but also presumably with a C-terminal truncation. Finding released SASP gamma (SspE) protein in the supernatant was equally a surprise. These SASP proteins, reported to be degraded within the spore core in normal germination, are being released from the cellular core of the spore by some unknown mechanism during this incomplete germination process. Presumably they are protected from specific germination protease (gpr) -mediated proteolysis by the failure to rehydrate the spore core, and hence activate this protease, in spores of this strain.

The degradation of the SASPs in the spore core is one of the later stages in the germination of *Bacillus* spores. It appears that aberrantly processed forms of SASPs are becoming trapped within the altered *cwID* spore coat during germination, in circumstances where the spore core does not rehydrate fully. **Our data suggest that there has been significant release of SASPs from the spore core, across the inner spore membrane, during the partial germination seen in a *cwID* mutant.**

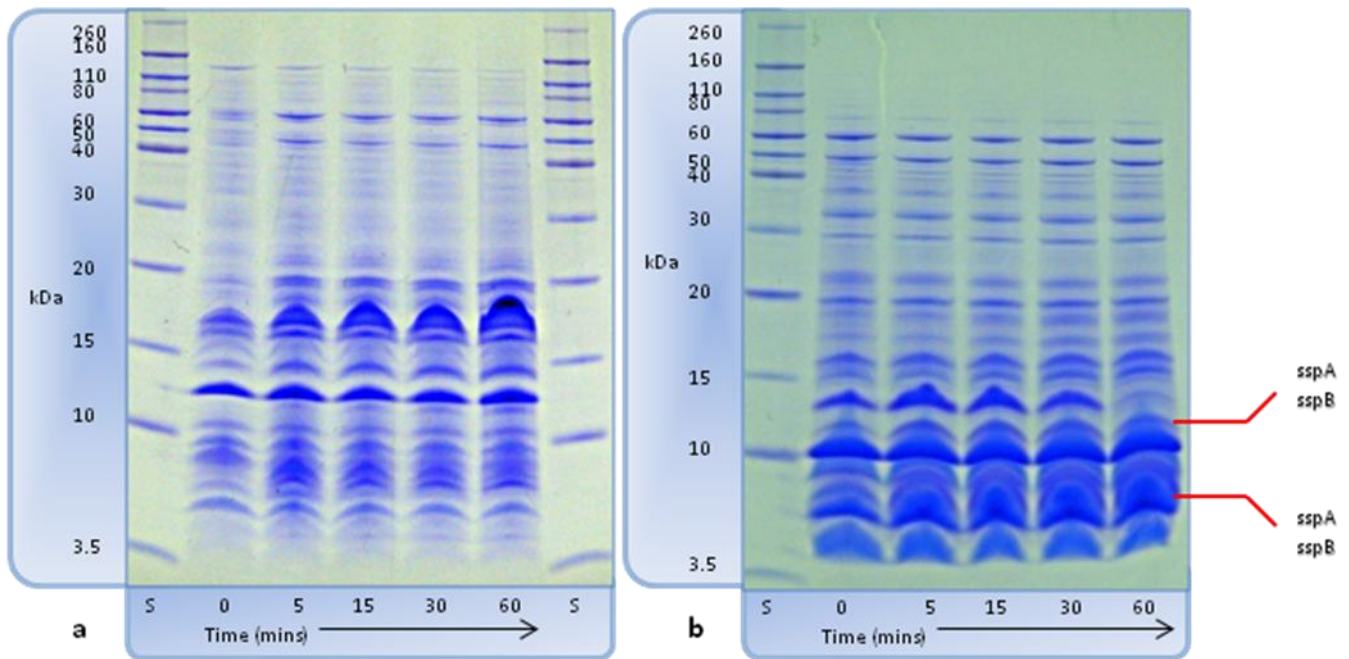


Fig. 2: Tris-Tricine gel profiles of changes in *B. subtilis* (wild-type) and *cwID* mutant spore coat proteins during germination. Protein profiles of coat extracts of germinating (a) *B. subtilis* (wild-type) and (b) *cwID* mutant spores (10 mM L-alanine at 37 °C). Samples taken at 0, 5, 15, 30 and 60 mins, boiled in neutral polyacrylamide gel electrophoresis lithium dodecyl sulphate (NuPAGE LDS) buffer and run on a Tris-tricine (16 %) gel. Profiles blotted onto Polyvinylidene Fluoride (PVDF) in N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer, and N-terminal sequencing performed on highlighted bands. S – Novex sharp standard.

Proteases in *Bacillus subtilis* spore coats. The presence or otherwise of proteases in dormant spore coats has been a neglected area of spore biology, and has been examined in this project. Two major proteases extracted from the *Bacillus subtilis* spore coat can be detected by zymography following renaturation after SDS-PAGE, using casein or gelatin as substrates. A 30kDa protease, that is resistant to heating in Tris buffer at 65 degrees, but is inactivated by heating at 65 °C in extraction buffer, remains in the spore coat during germination. In contrast, a 36kDa protease is more thermostable, remains active after extraction at 65 °C, and is released from the spore coat into the supernatant during germination, both as a monomer and in higher molecular weight-bound forms.

The two spore proteases are still present and active in a mutant lacking six extracellular proteases (NprE, AprE, Mpr, Bpf, NprB and Epr), indicating that they do not correspond to any of these. The 36 kDa protease is likely to be AprX, as the activity is missing from spores of an *aprX* mutant. AprX is a serine protease of the subtilase superfamily (Valbuuzzi et al., 1999). In *aprX* mutant strains, confirmed after transfer of the mutation into a fresh background, the other, 30kDa, protease appears to be overexpressed, perhaps in a compensatory manner. We have not yet succeeded in identifying the 30kDa protease, which is likely to be novel.

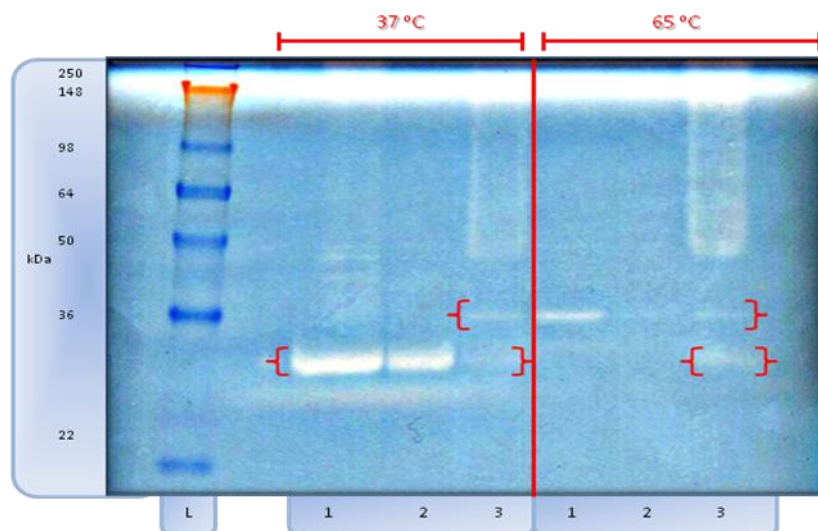


Fig. 3: Protease activity in *B. subtilis* (wild-type) dormant and germinated spore coats, and germination exudate. Coat protein was extracted from dormant and from germinated *B. subtilis* wild-type spores (2 h germination in Tris-HCl [pH 8.0 / 100 mM], 50 mM KCl, 100 mM L-alanine). Germination exudate was filtered through Millex GP 0.22 μ m filters (Millipore, U.S.A) then concentrated in Vivaspin 20 columns (Sartorius, Germany). Extracts were analysed on a Zymogram (4-12 % acrylamide) casein gel. Lane 1 –coat extract from dormant spores; lane 2 – coat extract from germinated spores; lane 3 – germination exudate. Extraction (or in the case of germination exudate, preincubation) was carried out at 37 °C or 65 °C. L- SeeBlue standards (Invitrogen, UK). Red brackets {} highlight protease activity.

***Bacillus borstelensis* spore properties.** A sporeformer with a spore coat that was exceptionally resistant to alkaline extraction (Bacillus X) was identified as *B. borstelensis* by rRNA gene sequencing. Analysis of 16S rDNA by PCR, amplified using the universal primers DG74 and RW01 (Greisen *et al.* 1994), revealed a 99 % similarity to previously described *Brevibacillus borstelensis*. Spores of this strain germinated very slowly and asynchronously, and it was not practical to undertake studies of the germination-associated coat changes in this species. Although the spore coat was resistant to alkali extraction, proteins were successfully solubilised, both from purified spore coat or from whole spores, by the method of Henriques *et al* (1995), which involved boiling for 8 min in a highly reducing buffer containing 4% LDS, 10% 2-mercaptoethanol, 1 mM dithiothreitol, and 125 mM Tris-HCl [pH 6.8]. The spore protein profile was identical to that of a standard strain of *Brevibacillus borstelensis*.

Conclusion

One major discovery in this project has been something of a negative one – most spore coat protein is slowly degraded in situ rather than being released. Nevertheless, minor changes in protein profiles of spore coat material, and release of some proteins into the supernatant, do both occur. We have deliberately used N-terminal sequencing to provide an identification of the major components represented in a complex protein profile, and have identified proteins released in wild type spores and in a *cwlD* mutant, in which cortex is not hydrolysed and enzyme activity does not resume in the spore core (Cowan et al., 2003). Both have yielded novel results.

Little is known of the presence of proteases in the spore. We have demonstrated a number of activities, by zymography, identified one as AprX, and characterised another, tightly spore-bound, protease of 30kDa which is not yet identified. It is likely that these proteases are important in breakdown of spore coat proteins, but we need to identify the second and generate double mutants in order to explore further their role in spore coat breakdown.

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