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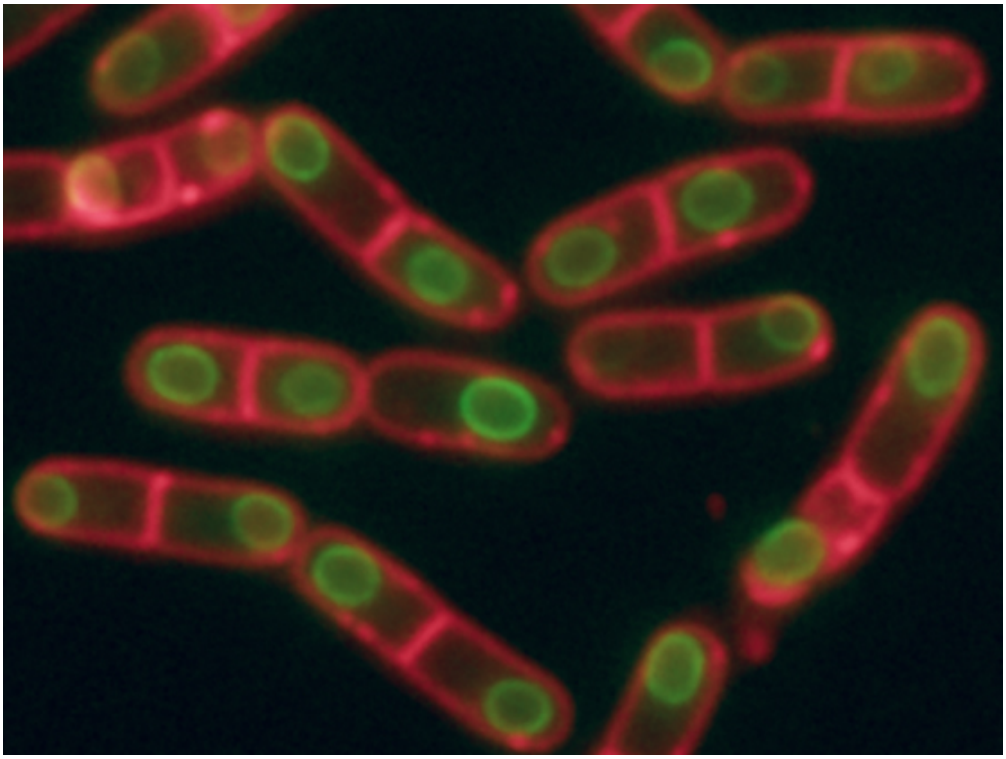
Journal:	*FEMS Microbiology Letters*
Manuscript ID	FEMSLE-19-03-0180.R1
Manuscript Type:	Research Letter
Date Submitted by the Author:	n/a
Complete List of Authors:	Al-Riyami, Bahja; University of Cambridge, Chemical Engineering and Biotechnology Ghosh, Abhinaba; University of Cambridge, Chemical Engineering and Biotechnology Rees, Eric; University of Cambridge, Chemical Engineering and Biotechnology Christie, Graham; University of Cambridge, Chemical Engineering and Biotechnology
Keywords:	</i>Bacillus</i>, spore germination, cortex lytic enzyme, peptidoglycan lysin
All articles in FEMS Microbiology Letters are published under one of eight subject sections. Please select the most appropriate subject category for your submission from the drop down list:	Physiology & Biochemistry (Editor: Jana Jass)



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Novel cortex lytic enzymes in *Bacillus megaterium* QM B1551 spores

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One sentence summary: Proteins encoded at BMQ_2391 and BMQ_3234 encode novel cortex lytic enzymes that permit cortex hydrolysis in *Bacillus megaterium* spores deficient in the major peptidoglycan lysins SleB and CwlJ.

Abstract

Present models for spore germination in *Bacillus* species include a requirement for either the SleB or CwlJ cortex lytic enzymes to efficiently depolymerise the spore cortex. Previous work has demonstrated that *B. megaterium* spores may differ to other species in this regard, since *sleB cwlJ* null mutant spores complemented with the gene in *trans* for the non-peptidoglycan lysin YpeB can efficiently degrade the cortex. Here we identify two novel cortex lytic enzymes, encoded at the BMQ_2391 and BMQ_3234 loci, that are essential for cortex hydrolysis in the absence of SleB and CwlJ. Ellipsoid localisation microscopy places the BMQ_3234 protein within the inner-spore coat, a region of the spore that is populated by other cortex lytic enzymes. The findings reinforce the idea that there is a degree of variation in mechanisms of cortex hydrolysis across the *Bacillales*, raising potential implications for environmental decontamination strategies based upon targeted inactivation of components of the spore germination apparatus.

Keywords: *Bacillus*, spore germination, cortex lytic enzyme, peptidoglycan lysin

Introduction

Bacteria of the *Bacillales* and *Clostridiales* form metabolically dormant endospores (spores) in response to nutrient limitation. The cellular properties of spores, which include several unique morphological and physiological features, means that they are equipped to persist in the environment, in a dormant state, until conditions are conducive to support vegetative growth. The latter is indicated by the presence of nutrient molecules, typically amino acids, monosaccharides and nucleosides, which interact with germinant receptor molecules to trigger spore germination (Setlow *et al.* 2017). A major event in spore germination concerns depolymerisation of the spore cortex, a thick layer of structurally unique peptidoglycan that envelops the spore protoplast and which is a major contributory factor to the maintenance of spore dormancy (Imae & Strominger 1976; Popham & Bernhards 2015). In spores of all species examined the cortex is degraded by specialised peptidoglycan lysins, which are referred to as cortex lytic enzymes (CLEs). It is well established that two main CLEs, SleB and CwlJ, can initiate hydrolysis of the cortex during the germination process (Moriyama *et al.* 1996; Ishikawa *et al.* 1998). SleB and CwlJ are semi-redundant enzymes, in the sense that deletion of either gene permits cortex hydrolysis to an extent where at least a proportion of the spores within a population will retain viability on rich culture medium. Deletion of both genes, however, results in spores that are severely compromised in their capacity to depolymerise the cortex resulting in arrested germination and severely reduced colony forming ability (Ishikawa *et al.* 1998; Heffron *et al.* 2009; Setlow *et al.* 2009).

As with other species of *Bacillus* where CLEs have been characterised by mutational analysis, *Bacillus megaterium* spores that are null for *sleB* and *cwlJ* lose absorbance (A600 nm) when suspended in buffer supplemented with germinants (Setlow *et al.* 2009). A reduction in A600 of approximately 15% is indicative of spores that have released calcium dipicolinate (CaDPA) from the spore core but cannot proceed to cortical depolymerisation and subsequent core hydration (for reference, the A600 of wild type spore suspensions decreases by approximately 60% when germinated under similar circumstances). Furthermore, the viability of *B. megaterium sleB cwlJ* spores is reduced by several orders of magnitude compared to the isogenic wild type strain. Unexpectedly, the introduction on a low copy number plasmid of a truncated *sleB* gene, encoding only the N-terminal non-catalytic domain, plus *ypeB*, which resides in the same operon as *sleB*, restores spore viability and absorbance loss to near wild type levels in the *sleB cwlJ* spores background (Christie *et al.* 2010). Structural analysis of cortical fragments produced during

germination of these spores, and in spores where only *ypeB* was complemented, which also germinate efficiently (Ustok *et al.* 2014), revealed a relative abundance of anhydromuropeptides in the germination milieu. These are the products of a class of peptidoglycan lysin referred to as a lytic transglycosylase. Since SleB is a well characterised lytic transglycosylase (Boland *et al.* 2000; Jing *et al.* 2012; Li *et al.* 2012), the obvious conclusion here is that an orthologue of SleB is present in *B. megaterium* spores, which – like SleB - has an undefined requirement for YpeB, and which can initiate cortex depolymerisation in the absence of SleB and CwlJ.

Accordingly, the objective of the current study was to identify and characterise novel CLEs that are functional during the germination of *B. megaterium* QM B1551 spores, and in particular, those that permit efficient cortex hydrolysis in the absence of SleB and CwlJ.

Materials and Methods

Bacterial strains and spore preparation

B. megaterium strains employed in this study, which were all isogenic with the QM B1551 strain, were cultured routinely at 30°C on LB medium supplemented where appropriate with antibiotics (Table 1). Spores were prepared by nutrient exhaustion in supplemented nutrient broth and subsequently purified by repeated rounds of centrifugation and resuspension of spore pellets in deionised ice-cold water (Christie *et al.* 2010). Purified suspensions comprising >99% phase bright spores were stored in deionised water, at an A600 of approximately 50, on ice. *Escherichia coli* Top 10 cells (Thermo Fisher, UK) were used for cloning procedures, plasmid propagation and for heterologous protein expression experiments.

Spore germination and viability assays

Spore germination in liquid medium was assessed in microtitre plates by adding 10 µl of heat-shocked (80°C, 30 min) spores to 190 µl of 5% (wt/vol) beef extract (Oxoid, Ltd., Basingstoke, United Kingdom), preheated to 37°C. The A600 of spore suspensions was 0.4. Plates were sealed with adhesive film to minimize evaporative losses, and then incubated in a Perkin-Elmer EnVision-Xcite multilabel plate reader fitted with a 600 nm photometric filter. Plates were agitated orbitally for 10 seconds prior to absorbance measurements, which were recorded every minute for 90 minutes. Experiments were conducted in triplicate, with at least two or more independent spore preparations. Spore viability was determined by pipetting 10 µl aliquots of serially diluted

suspensions of heat-shocked spores (A600 of 1 [10^8 spores/ml]) onto LB agar plates. Plates were incubated for 16 to 24 h prior to colony enumeration.

Molecular biology procedures

Transcriptional analysis from loci of interest was examined by RT-PCR, using RNA purified from sporulating cultures, a QuantiTect Reverse Transcription kit (Qiagen, Manchester, UK) and gene-specific primers designed to amplify approximately 400 bp fragments, essentially as described previously (Ramirez-Peralta *et al.* 2013).

Inactivation of BMQ_2391 and BMQ_3234 in the *B. megaterium sleB cwlJ* background was accomplished by integrating pUCTV2 derived plasmids at the respective loci. Essentially, the first 300 bp of each gene was amplified from *B. megaterium* QM B1551 genomic DNA by PCR. Purified PCR products were ligated with pUCTV2 (digested with EcoRI), and ligation mixtures subsequently used to transform *E. coli* to carbenicillin resistance. Isolated plasmids were introduced to *B. megaterium* protoplasts using standard polyethylene glycol mediated transformation procedures. Tetracycline resistant transformants were then repeatedly streaked on LB agar plates containing 1.25 µg/ml tetracycline at 42°C to permit isolation of clones that had integrated plasmid DNA at the cloned loci, prior to validating by PCR and sequencing. Strains with translational *gfp* fusions to genes of interest were constructed by amplifying entire BMQ_2391 and BMQ_3234 ORFs, minus stop codons, using primers that incorporated XhoI and EcoRI restriction sites at the respective 5' and 3' ends. Purified and digested PCR amplicons were ligated with similarly digested pVLG6 vector to create 3' fusions to *gfp*. The resultant plasmids were purified from *E. coli* and used to transform *B. megaterium* to chloramphenicol resistance. Individual colonies were streaked and incubated at the non-permissive temperature of 42°C in the presence of chloramphenicol, to select for plasmid integrations into the chromosome at cloned loci. Procedures designed to create spores with transcriptional fusions between putative CLE promoter sequences and *lacZ* were constructed using plasmid pNFd13, and resultant measurements of β-galactosidase activity associated with disrupted spores, were as described previously (Gupta *et al.* 2013).

Complementation-based analyses of CLE mutant strains were performed using pHT315-derived plasmids, which have a copy number of approximately 15 per cell (Arantes & Lereclus 1991). The existing pHT-*sleB*^{N} *ypeB* plasmid (Christie *et al.* 2010) was modified to additionally

incorporate ORFs and upstream regulatory sequences for either BMQ_2391 or BMQ_3234 proteins. This was achieved using EcoRI restricted pHT-*sleB*^{N} *ypeB* and BMQ_2391 or BMQ_3234 PCR amplicons designed to facilitate cloning via the Klenow Assembly Method (Ghosh *et al.* 2018). Similar procedures were used to introduce BMQ_2391 or BMQ_3234 ORFs and regulatory sequences into pHT315. The *B. megaterium sleB cwlJ BMQ_2391* and *B. megaterium sleB cwlJ BMQ_3234* strains were subsequently transformed, using the appropriate plasmids, to erythromycin and lincomycin resistance.

Ellipsoid localisation microscopy

The quantitative fluorescence ELM technique was used to measure the location of GFP fusion proteins in mature spores (Manetsberger *et al.* 2015; Manton *et al.* 2018). Briefly, several independent fields of GFP labelled spores were imaged using an Olympus BX53 microscope fitted with a 100X 1.30 NA oil objective lens, with illumination from a mercury lamp, filters for GFP fluorescence, and a Retiga 2000R CCD camera. Automated image segmentation was used to identify single spores, and the image of each candidate was used to fit the parameters of a model that describes the image of a spheroidal fluorescent shell. For *B. megaterium*, an equation describing the image of a spherical fluorescent layer was fitted to the image data. A filter was applied to exclude fits from overlapping spores and fragments of debris. The average radius parameter fitted to the spores provides an estimate of the midpoint radial position of the GFP fusion with respect to the spore centre.

Protein interactions

A bacterial adenylate cyclase two-hybrid (BACTH) system (Euromedex, Souffelweyersheim, France) was used to characterise potential interactions *in vivo* between *B. megaterium* CLEs and related proteins (BMQ_2391, BMQ_3234, CwlJ, SleB, SleL and YpeB). The BACTH system is based upon two complementary adenylate cyclase fragments (T25 and T18) being brought together by interacting fusion partners to form functional enzyme, resulting in cAMP synthesis and activation of a *lacZ* reporter gene. Plasmids used for BACTH assays in this work were prepared by PCR amplifying ORFs encoding CLEs and YpeB from genomic DNA before assembling with linearised pUT18 and pKT25 plasmids by Klenow assembly. The resulting plasmids, designed for expression of C-terminal fusions between proteins of interest and T25 or T18 adenylate cyclase

fragments, were isolated and verified by sequencing. Electrocompetent *E. coli* BTH101 were co-transformed using various combinations of pKT25 and pUT18 derived plasmids and selected on LB agar plates supplemented with 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 1 mM IPTG, and 50 µg/ml carbenicillin and kanamycin, respectively. Cells were cultured for 24-72 h at 30°C and examined for the development of blue coloration, which is indicative of positive interactions between pairs of fusion proteins.

Results

Identifying potential CLE genes

Genes encoding candidate novel CLEs were identified by conducting NCBI Protein BLAST searches using sequences for the C-terminal catalytic domain of SleB and full length CwlJ as probes against the *B. megaterium* QM B1551 genome (GenBank accession numbers CP001983 – CP001900). The searches identified five potential CLEs, four of which showed greater amino acid sequence identity with the catalytic domain of SleB (ranging from 40 – 46 %), whereas BMQ_3234 shared greater sequence identity to CwlJ (49%) (Table 2). Sequence analysis revealed possible sigma G (σ^G) consensus sequences and predicted signal peptide sequences for all four SleB homologues, which is consistent with expression in the forespore during sporulation. In contrast, the BMQ_3234 ORF is preceded by a potential sigma E (σ^E) recognition sequence and lacks a recognisable signal peptide, which is consistent with mother cell expression in the mother cell during sporulation. All five predicted proteins are members of the Hydrolase-2 family (PF07486), which is consistent with a role in peptidoglycan depolymerisation. Two of the putative proteins contain single peptidoglycan-binding LysM domains (BMQ_1284 and BMQ_2145) with BMQ_3195 predicted to have two N-terminal LysM domains. LysM domains are present in established spore CLEs, including SleL (Ustok *et al.* 2015). In contrast, neither BMQ_2391 nor BMQ_3234 appear to contain defined substrate binding domains, which is analogous to the domain architecture of CwlJ. Pairwise sequence alignments indicate that SleB's single catalytic residue, E208, is present and conserved in the primary sequence of all five putative CLEs (Table 2).

Transcriptional analysis of candidate CLE genes

In order to ascertain whether the various candidate CLE genes were transcribed during sporulation,

RT-PCR was conducted on cDNA derived from *B. megaterium* cultures sampled immediately prior to entry to stationary phase and at hourly intervals thereafter (Figure 1). These analyses indicate that two of the candidate CLEs - BMQ_1284 and BMQ_2145 – are transcribed at very low levels based on the low abundance of RT-PCR products. BMQ_2391 is transcribed from the early to mid-stages of sporulation, with a pattern of expression that most closely matches that of *sleB*. In contrast, BMQ_3195 transcripts are most abundant from the mid to latter stages of sporulation, whereas BMQ_3234 appears to be transcribed strongly throughout. The latter pattern of transcription matches that of *cwlJ*. Further transcriptional analyses of candidate and established CLE promoters were achieved by placing the *lacZ* gene under control of putative promoter sequences for the various genes. The outputs from β-galactosidase assays conducted with supernatants from disrupted spores of the various *lacZ* reporter strains are broadly in line with results from the aforementioned RT-PCR assays i.e. where BMQ_1284 and BMQ_2145 transcriptional fusions are associated with little β-galactosidase activity, while BMQ_2391 and BMQ_3234 show the highest levels of expression, albeit less than evident for *sleB* and *cwlJ* transcriptional fusions (Table 1).

Localisation of BMQ_2391 and BMQ_3234 GFP fusion proteins

Proteins encoded at the BMQ_2391and BMQ_3234 loci were selected for further analysis since they appeared to be expressed at the highest levels of the candidate CLEs. The sub-cellular localisation of both proteins was investigated during sporulation and in mature spores by creating constructs designed to express C-terminal GFP fusion proteins. Fluorescence microscopy revealed that the BMQ_3234-GFP protein is expressed during sporulation in the mother cell compartment and is deposited to form a ring of fluorescence around the developing forespore (Figure 2). A strong fluorescence signal was retained in mature spores (Figure 2). The average radial location of the BMQ_3234-GFP protein, as inferred from ellipsoid localisation analyses, was 521 nm ±9 nm in mature spores (where ±9 nm is the standard deviation of radial locations found in repeated measurements and where 865 spores were analysed with a residual fitting error of 8.4%). This places the location of BMQ_3234 to the inner spore coat, within the same vicinity as SleL (525 nm ±11 nm; 362 spores analysed with a residual fitting error of 6.5%), and presumably CwlJ (Setlow *et al.* 2017; Ghosh *et al.* 2018). In contrast, no fluorescence was observed during sporulation or in mature spores for the BMQ_2391-GFP protein, perhaps due to aberrant protein

folding or problems associated with the GFP fusion protein translocating the inner membrane (data not shown). A strain designed to express an N-terminal GFP fusion protein also failed to show any detectable fluorescence during sporulation or in mature spores.

Germination of BMQ_2391 and BMQ_3234 null mutant spores

To investigate whether the BMQ_2391 and BMQ_3234 proteins have a role in germination, particularly in the absence of the major CLEs, mutant strains lacking either of these enzymes were constructed in the *B. megaterium sleB cwlJ* double mutant background. Despite repeated attempts we failed to isolate mutants that had undergone allelic exchange with truncated and disrupted gene variants at either locus. Instead, strains that had undergone single recombination events, integrating pUCTV2 derived plasmids at the target loci and thereby separating the respective ORFs from their promoter sequences, were isolated. Copies of *sleB*^{N} and *ypeB* under control of the native promoter sequence were introduced to these strains on a low copy number pHT315 derived plasmid, and the resultant strains sporulated by nutrient starvation. Both strains were found to have excised the pBM600 plasmid during mutagenesis. This plasmid encodes the GerU-germinant receptor, hence the resultant spores were heat shocked and germinated in beef extract medium, which promotes an efficient germinative response in the absence of GerU (Gupta *et al.* 2013). Germination assays conducted with spores suspended in beef extract revealed that both triple mutant strains - *sleB cwlJ BMQ_2391* and *sleB cwlJ BMQ_3234* - complemented with plasmid bourne *sleB*^{N} and *ypeB*, had major germination defects, with the A600 decreasing by a comparable amount (~15 %) to *sleB cwlJ* spores (Figure 3). This is indicative of CaDPA efflux but limited, if any, cortex depolymerisation. The viability of both triple mutant strains was also severely compromised compared to wild type and *sleB cwlJ* spores complemented with *sleB*^{N} *ypeB*, again being comparable to *sleB cwlJ* spores lacking plasmid borne *sleB*^{N} *ypeB* (Table 1). In contrast, the viability of triple mutant strains complemented with plasmid borne copies of *sleB*^{N} *ypeB* and *BMQ_2391* or *BMQ_3234*, as appropriate, was restored to approximately half of that observed in *sleB cwlJ* pHT-*sleB*^{N} *ypeB* spores (35-40 % versus 80 %). Similarly, complemented spores show a reduction in A600 of ~50 % within 40 minutes in beef extract, which although not as efficient as *sleB cwlJ* pHT-*sleB*^{N} *ypeB* spores, is indicative of significant cortical depolymerisation (Figure 3). In contrast, the viability of triple mutant spores complemented with either BMQ_2391 or

BMQ_3234 was comparable to spores of the respective non-complemented strains, indicating that YpeB is essential for cortical depolymerisation in the *sleB cwlJ* background.

Interactions between CLEs

Results with null mutant spores reported in this work, and in previous studies (Bernhards & Popham 2014; Ustok *et al.* 2014), indicate that direct physical interactions between CLEs and or CLEs and the YpeB protein may be required to facilitate efficient cortex hydrolysis during germination. The *E. coli* based BACTH system was employed in the current study to investigate potential interactions between the two novel CLEs identified in this work – BMQ_2391 and BMQ_3234 – with each other and with SleB, CwlJ, SleL and YpeB. Potential interactions between (i) SleB with YpeB, SleL and CwlJ, (ii) CwlJ with YpeB and SleL, and (iii) SleL with YpeB, were also examined. Blue coloration of co-transformant *E. coli* colonies was not observed in any of the combinations tested, with the exception of the positive control, indicating that none of the spore proteins of interest interact productively in this system (Figure 4).

Heterologous expression of BMQ_2391 and BMQ_3234

Several attempts were made to express recombinant versions of the BMQ_2391 and BMQ_3234 proteins with a view to characterising the hydrolytic bond specificity of each protein. Hosts for heterologous expression included *E. coli*, *Lactococcus lactis* and *B. subtilis*, with several variant proteins – including truncated forms, GFP, and maltose binding protein (solubility enhancer) fusions – being examined over the course of this work. Unfortunately, levels of expression commensurate with biochemical analyses were not achieved in any of the systems employed (data not shown).

Discussion

Initiation of cortex hydrolysis in current models of *Bacillus* spore germination requires the activity of either of the semi redundant CLEs SleB or CwlJ. Efficient degradation of the cortex is subsequently facilitated by enzymes such as SleL, whose function appears to be associated with further hydrolysis of large peptidoglycan fragments generated by SleB and or CwlJ (Chen *et al.* 2000; Lambert & Popham 2008; Ustok *et al.* 2015). While this sequence of events probably occurs in wild type spores of all species of *Bacillus*, mutagenesis analyses conducted with *B. megaterium*

sleB cwlJ spores have revealed that this species can circumvent the requirement for either of SleB or CwlJ . Previous work revealed that *B. megaterium sleB cwlJ* spores could degrade the cortex with an efficiency comparable to wild type spores when complemented with a plasmid-borne copy of $sleB^N$, which encodes the non catalytic domain of SleB, plus the adjacent *ypeB* gene (Christie *et al.* 2010). Indeed, complementation with *ypeB* alone restored efficient cortex hydrolysis to *sleB cwlJ* spores (Ustok *et al.* 2014). The precise function of YpeB has not been determined, although it is not a peptidoglycan lysin, and instead appears to have a role in localising – and perhaps regulating the activity - of SleB in the spore (Boland *et al.* 2000; Bernhards & Popham 2014; Ustok *et al.* 2015).

Another study in *B. megaterium* extended these findings and attempted to dissect the molecular mechanisms that support efficient cortex hydrolysis in *sleB cwlJ* spores (Ustok *et al.* 2014). In this case, a triple mutant (*sleB cwlJ sleL*) strain complemented with $sleB^N$ plus *ypeB* showed a severe germination defect. Based on this result, the study concluded that *ypeB* and *sleL* are both essential for the initiation of cortex hydrolysis in *B. megaterium sleB cwlJ* spores. The requirement for *sleL* in the *sleB cwlJ* background was unexpected since, as noted above, SleL is regarded as a cortical fragment lytic enzyme. Similarly, muropeptide analyses of germination exudates from *sleB cwlJ* spores complemented with *ypeB* clearly indicate the presence of lytic transglycosylase activity during germination, whereas SleL exhibits N-acetylglucosaminidase activity (Lambert & Popham 2008; Ustok *et al.* 2014).

With this context in mind, the main purpose of the current study was to identify cortex lytic enzymes that are functional in *B. megaterium sleB cwlJ* spores complemented with plasmid borne *ypeB*. Progress in this regard was achieved via bioinformatic analyses, which identified five candidate CLE loci. Subsequent transcriptional analyses, comprising RT-PCR and β-galactoside reporter assays, were used to narrow candidates for further characterisation to proteins encoded at the BMQ_2391 and BMQ_3234 loci (moderate transcription, relative to *sleB* and *cwlJ*, was associated with BMQ_3195, although this protein was not characterised further).

Mutagenesis analyses in this work were compromised to a certain extent in that we could not isolate null mutant strains that had undergone allelic exchange with truncated and disrupted versions of *BMQ_2391* or *BMQ_3234* in the *sleB cwlJ* background. However, strains in which *BMQ_2391* or *BMQ_3234* were disrupted by integrative plasmids, effectively separating the promoter and first 300 nucleotides of the respective genes from the remainder of the coding

sequences, were isolated, albeit at the expense of the GerU-encoding pBM600 plasmid. Loss of the GerU germinant receptor can be circumvented by germinating *gerU* spores in beef extract, components of which trigger efficient germination responses via alternative germinant receptors in *B. megaterium* (Gupta *et al.* 2013). In both cases, germination of triple mutant spores – *sleB cwlJ BMQ_2391* and *sleB cwlJ BMQ_3234* – complemented with plasmid borne $sleB^N$ and *ypeB*, was comparable in terms of absorbance loss to non $sleB^N$ *ypeB* complemented triple mutant spores. The observed reduction in A600 of approximately 15% is indicative of spores that have released CaDPA and various ions from the spore core but which have failed to depolymerise cortical peptidoglycan. Viability of the $sleB^N$ *ypeB* complemented triple mutant spores was similarly comparable to non-complemented spores, with colony forming ability on LB medium being reduced by more than five logs compared to isogenic wild type spores, indicating again that the cortex has not been degraded. In contrast, complementation in trans with *BMQ_2391* or *BMQ_3234* in $sleB^N$ *ypeB* complemented triple mutant spores restored viability and absorbance loss to approximately 40% of wild type levels.

Outputs from the present study support the hypothesis that *BMQ_2391* and *BMQ_3234* encode novel CLEs that are not only active during germination of *B. megaterium* spores, but which also confer further redundancy within the cortical depolymerisation system of this species (and perhaps this species alone since $sleB^N$ *ypeB* in trans failed to restore cortex hydrolysis to *B. subtilis sleB cwlJ* spores (Li *et al.* 2013)). In some regards the requirement for three separate enzymes - SleL, BMQ_2391 and BMQ_3234 – in tandem with YpeB, for efficient spore germination in *B. megaterium sleB cwlJ* spores, is difficult to reconcile. One possibility is that the combined activity of each enzyme – which may be infrequent cutters – is required for cortex depolymerisation to a degree that is commensurate with germination. It's possible also that at least some of these proteins physically interact to ensure correct localisation and or function in the spore. Ellipsoid localisation microscopy analysis places SleL and BMQ_3234 within the same inner coat location within the spore, which would facilitate physical interactions. However, where examined - bacterial two-hybrid assays in this work and pull-down assays conducted previously (Li *et al.* 2013; Ustok *et al.* 2014) – have failed to provide any evidence of interactions between CLEs. Further insight to the nature of the inter-dependency between CLEs, and how YpeB may contribute to this, are objectives for continuing work in this area. Finally, targeted inactivation of CLEs may provide a novel strategy for spore decontamination in a number of sectors. A potential implication arising

from the present study is that enzymes in addition to the major CLEs may have to be inhibited, at least in some species, for efficient spore inactivation.

Acknowledgements

BAR was the recipient of a scholarship from the Sultanate of Oman government. AG was the recipient of a Cambridge Nehru Scholarship and received support from the Raymond and Beverly Sackler Foundation. Contributions to this work by Fatma Isik Ustok, Srishti Gupta, Ke Xu Zhou and Akshay Deshmukh are acknowledged.

Conflicts of interest

None declared

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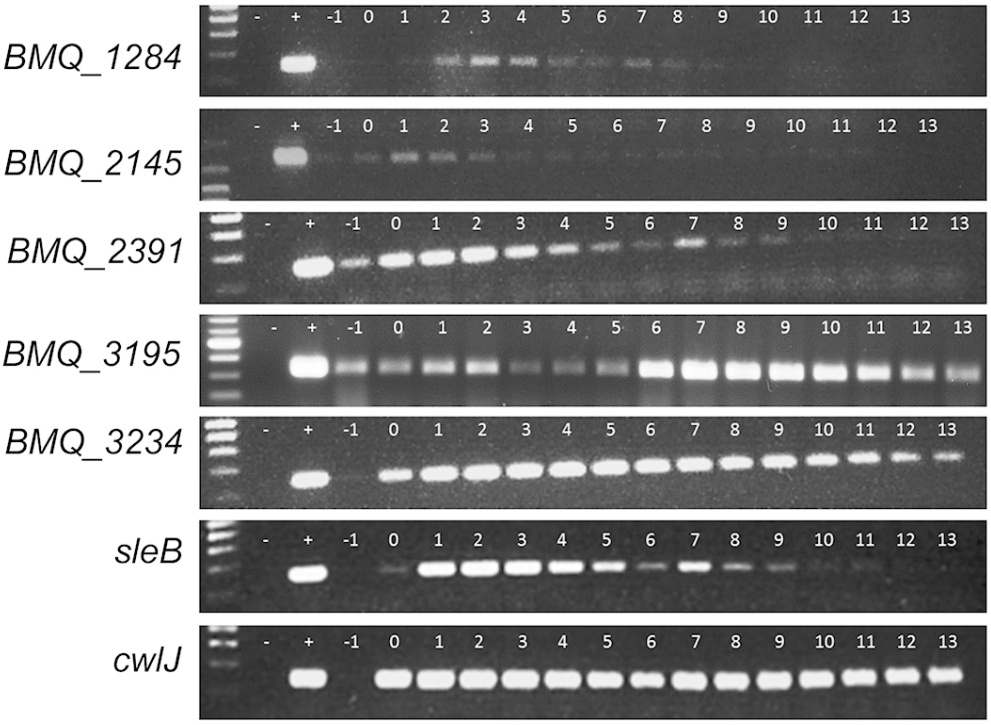
Figure legends

Figure 1 RT-PCR analysis of *sleB*, *cwlJ* and potential CLE gene transcription during sporulation of *B. megaterium* QM B1551. RT-PCR was conducted using gene-specific primers designed to amplify ~300-bp fragments of the respective genes from RNA isolated from sporulating cultures, as described in Materials and Methods. Numbers refer to the times (h) after entry to sporulation. Negative, i.e., no template RNA (-), and positive, i.e. genomic template DNA (+), control reactions are indicated. Molecular weight markers are shown on the left-hand side. Isolated RNAs were verified as being free from genomic DNA by conducting PCRs with the same gene-specific primers (data not shown).

Figure 2 Phase contrast and fluorescence microscopy of *B. megaterium BMQ_3234-gfp* sporulating cells (a and b) and mature spores (c and d). Green fluorescence is associated with the BMQ_3234-GFP fusion protein and red fluorescence with the membrane-staining FM4-64 dye. Bar represents 5 µm.

Figure 3 Germination of *B. megaterium* spores in 5% (wt/vol) beef extract. Spores of the various strains were heat shocked (80^{o}C for 30 min) and then cooled before incubating in beef extract, and absorbance (A600) measurements recorded as described in the Materials and Methods. Key: GC103 (Δ*sleB* Δ*cwlJ*), crosses; GC106 (Δ*sleB* Δ*cwlJ* pHT315-*sleB*^{N} *ypeB*), diamonds; BR111 (Δ*sleB* Δ*cwlJ* Δ*BMQ_2391* pHT-*sleB*^{N} *ypeB*) filled squares; BR113 (Δ*sleB* Δ*cwlJ* Δ*BMQ_2391* pHT-*sleB*^{N} *ypeB BMQ_2391*), open squares; BR112 (Δ*sleB* Δ*cwlJ* Δ*BMQ_3234* pHT-*sleB*^{N} *ypeB*), filled circles; BR114 (Δ*sleB* Δ*cwlJ* Δ*BMQ_3234* pHT-*sleB*^{N} *ypeB BMQ_3234*), open circles.

Figure 4 Sample output from bacterial adenylate cyclase two-hybrid analysis for interactions between *B. megaterium* CLEs. Positive interactions, denoted by blue *E. coli* colonies, were observed only for the manufacturer's positive control (a). White colonies – indicative of proteins that do not interact - were observed for all CLE and YpeB pairings tested, including (b) SleB and SleL, and (c) BMQ_2391 and BMQ_3234.



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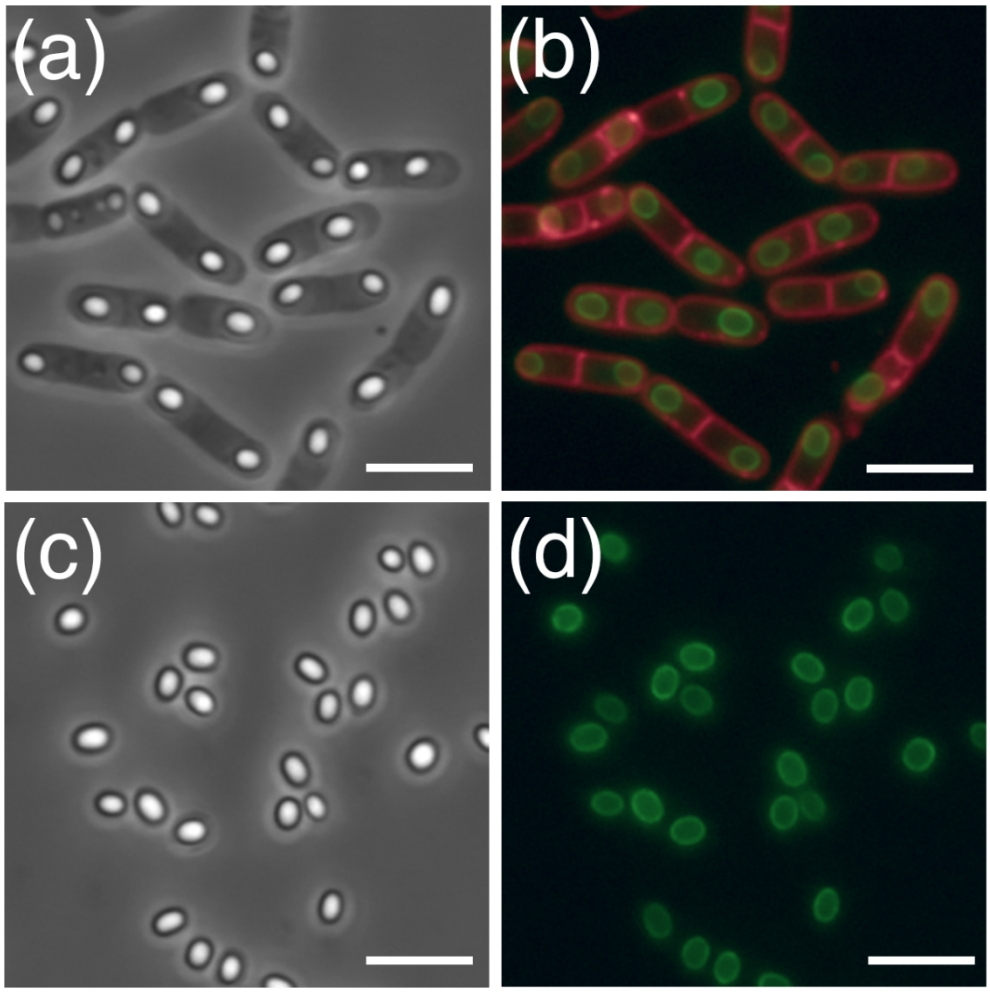
90x67mm (300 x 300 DPI)



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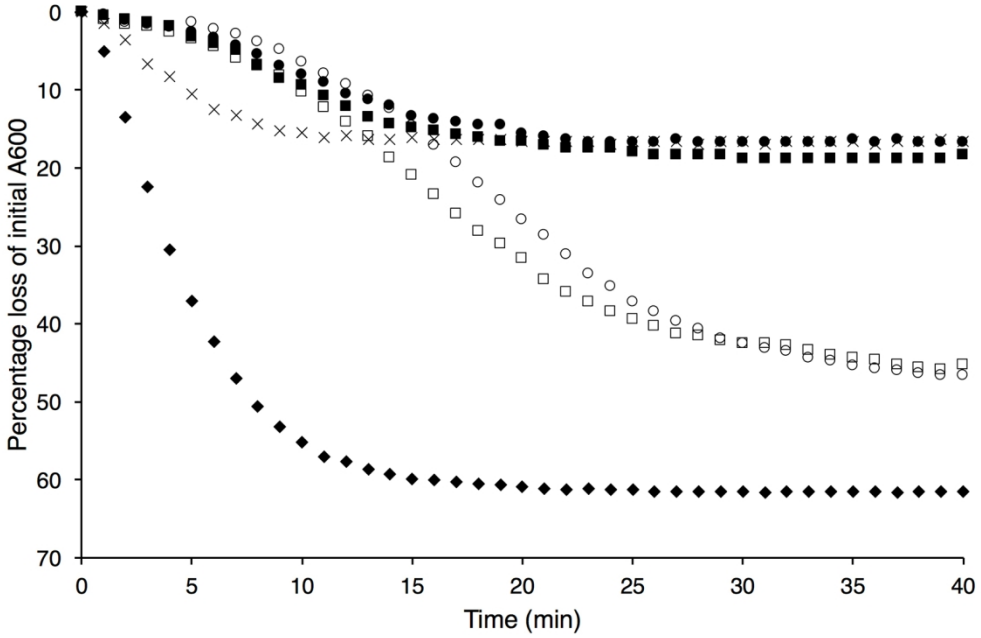
103x102mm (300 x 300 DPI)



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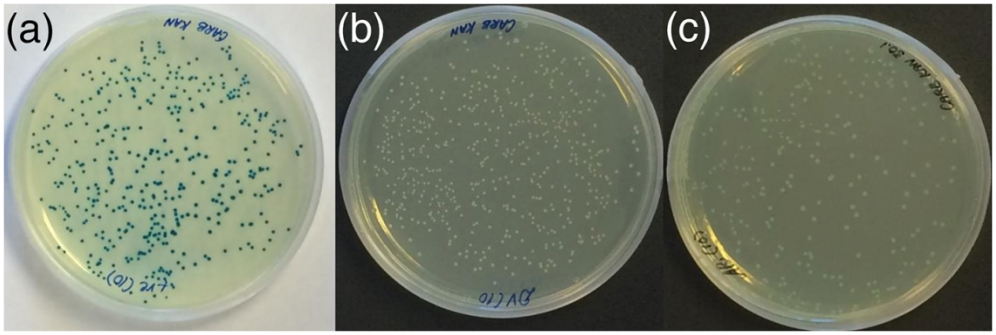
150x97mm (300 x 300 DPI)



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119x40mm (300 x 300 DPI)

Table 1 Strains of *Bacillus megaterium* used in this work and viability of their spores[a]

Strain	Relevant genotypic and phenotypic characteristics[b]	Viability (%)	β-Galactosidase activity (RFU)[c]	Source (reference)
QM B1551	Wild type	100	—[d]	Pat Vary
GC103	Δ*sleB* Δ*cwlJ* Km^r Sp^r	0.001	—	(Christie *et al.*, 2010)
GC106	Δ*sleB* Δ*cwlJ* pHT-*sleB*^{N} *ypeB* Km^r Sp^r MLS^r	80	—	(Christie *et al.*, 2010)
GC123	Δ*sleB* Δ*cwlJ* pHT-*ypeB* Km^r Sp^r MLS^r	50	—	(Ustok *et al.*, 2014)
BR111	Δ*sleB* Δ*cwlJ* Δ*BMQ_2391* pHT-*sleB*^{N} *ypeB* Km^r Sp^r Tc^r MLS^r	<0.001	—	This study
BR112	Δ*sleB* Δ*cwlJ* Δ*BMQ_3234* pHT-*sleB*^{N} *ypeB* Km^r Sp^r Tc^r MLS^r	<0.001	—	This study
BR113	Δ*sleB* Δ*cwlJ* Δ*BMQ_2391* pHT-*sleB*^{N} *ypeB BMQ_2391* Km^r Sp^r Tc^r MLS^r	40	—	This study
BR114	Δ*sleB* Δ*cwlJ* Δ*BMQ_3234* pHT-*sleB*^{N} *ypeB BMQ_3234* Km^r Sp^r Tc^r MLS^r	35	—	This study
BR115	Δ*sleB* Δ*cwlJ* Δ*BMQ_2391* pHT-*BMQ_2391* Km^r Sp^r Tc^r MLS^r	0.001	—	This study
BR116	Δ*sleB* Δ*cwlJ* Δ*BMQ_3234* pHT-*BMQ_3234* Km^r Sp^r Tc^r MLS^r	0.002	—	This study
			—	
lacZ fusion strains				
BR101	Δ*BMQ_1284*::pNFd13 Km^r	—	170	This study
BR102	Δ*BMQ_2145*::pNFd13 Km^r	—	210	This study
BR103	Δ*BMQ_2391*::pNFd13 Km^r	—	1115	This study

BR104	Δ*BMQ_3195*::pNFd13 Km^r	—	490	This study
BR105	Δ*BMQ_3234*::pNFd13 Km^r	—	1130	This study
BR106	Δ*BMQ_sleB*::pNFd13 Km^r	—	1950	This study
BR107	Δ*BMQ_cwlJ*::pNFd13 Km^r	—	1755	This study
gfp fusion strains				
BR108	*BMQ_2391-gfp* Cm^r	—	—	This study
BR109	*BMQ_3234-gfp* Cm^r	—	—	This study
BR110	*gfp-BMQ_2391* Cm^r	—	—	This study

[a] Spores of various strains were prepared, purified, and heat shocked, and their relative viability with respect to wild type spores was determined as described in Materials and Methods. All values shown are averages of results with two independent spore preparations and are 25% of the value shown.

[b] Abbreviations for antibiotic resistance: Km^r, kanamycin (5 μg/ml); Sp^r, spectinomycin (100 μg/ml); Tc^r, tetracycline (10 μg/ml); MLS^r, lincomycin (25 μg/ml) and erythromycin (1 μg/ml); Cm^r, chloramphenicol (5 μg/ml).

[c] Spores were purified and assayed for β-galactosidase activity as described in Materials and Methods. β-galactosidase values (relative fluorescence units [RFU]) are the average of triplicate measurements, with background values subtracted, conducted with two different spore preparations. Standard deviations for all values are <15%.

[d] —, not determined.

Table 2 Properties of *B. megaterium* putative spore cortex lytic enzymes

Locus identifier	Number of amino acids	Signal peptide cleavage site[a]	Predicted size (kDa)	Amino acid sequence identity with SleB[C] (%)[b]	Amino acid sequence identity with CwlJ (%)[b]	Predicted peptidoglycan binding domains[c]	Predicted catalytic glutamate[d]
BMQ_1284	233	28/29	22.3	46 (61)	24 (39)	LysM	E127
BMQ_2145	191	24/25	18.2	40 (63)	27 (39)	LysM	E90
BMQ_2391	152	24/25	14.1	43 (66)	27 (39)	none	E50
BMQ_3195	268	28/29	25.5	41 (60)	27 (40)	LysM (2)	E162
BMQ_3234	146	none	16.8	28 (46)	49 (67)	none	E21

[a] Predicted by the SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/abstract.php). Numbers refer to residue position in the primary amino acid sequence.

[b] Based on EMBOSS Water pairwise local sequence alignments (https://www.ebi.ac.uk/Tools/psa/emboss_water/). Values in brackets, % similarity.

[c] According to UniProt

[d] The EMBOSS Water sequence alignment tool indicates that these residues are conserved with catalytic E208 in *B. megaterium* SleB.

Response to Reviewers' comments

Novel cortex lytic enzymes in *Bacillus megaterium* QM B1551 spores

Manuscript id: FEMSLE-19-03-0180

Authors: Al-Riyami, Bahja; Ghosh, Abhinaba; Rees, Eric; Christie, Graham

The authors appreciate the time and effort taken by the reviewers in considering our manuscript and thank them for their useful suggestions, critiques and clarifications.

Reviewer: 1

This manuscript provides strong evidence on the identity of additional enzymes besides the two known redundant cortex-lytic enzymes CwlJ and SleB can give sufficient cortex peptidoglycan hydrolysis to allow completion of B. megaterium spore germination. There are, however, a number of relatively minor concerns that need to be addressed as follows.

1) There are a number of examples of unusual word usage in the manuscript. Three early in the manuscript are "initiate" on l 17, "consistent" on l 24 and "encompass" on l 31

Response
The abstract has been modified i.e. 'initiate' has been deleted, whereas the sentence with 'consistent' now reads 'The findings reinforce the idea that there is a degree of variation in mechanisms of cortex hydrolysis across the *Bacillales*, raising potential implications for environmental decontamination strategies based upon targeted inactivation of components of the spore germination apparatus.' Line 31 has been changed from 'encompass' to 'include'.

2) The abstract never mentions YpeB, yet this alone is sufficient to allow cwlJ sleB spore to germinate.

Response
The abstract has been modified to include the sentence: 'Previous work has demonstrated that *B. megaterium* spores may differ to other species in this regard, since *sleB cwlJ* null mutant spores complemented with the gene in *trans* for the non-peptidoglycan lysin YpeB can efficiently degrade the cortex.'

3) The issue of the possible implications of the additional CLEs shown in this manuscript to environmental decontamination is never mentioned again.

Response
A short section has been added to the end of the manuscript i.e. "Finally, targeted inactivation of CLEs may provide a novel strategy for spore decontamination in a number of sectors. A potential implication arising from the present study is that enzymes in addition to the major CLEs may have to be inhibited, at least in some species, for efficient spore inactivation.'

4) l 39/40 - A little misleading here, in that all Clostridial spores have CLEs, just many don't have CwlJ and SleB, but rather SleC.

Response

Correct, that was an unintended consequence from a previous edit. The sentence now reads ‘In spores of all species examined the cortex is degraded by specialised peptidoglycan lysins, which are referred to as cortex lytic enzymes (CLEs).’

5) l 56 - what is the copy number, and was the truncated SleB needed, and if not, what is the YpeB level needed to get cwlJ sleB spores to germinate?

Response
The copy number of pHT315-derived plasmids is approximately 15 per cell. We’ve added a reference to this effect in the manuscript. A significant number of sleB cwlJ spores germinate in the absence of truncated SleB, although the efficiency is decreased with respect to spores where both ypeB and SleBN are present. A citation to the relevant work is now included in line 61.

6) How was YpeB complemented and what was the level in complemented spores?

Response
YpeB was complemented using a pHT315-derived plasmid. A reference to this work is now cited in line 61.

7) l 119 - what is "low-copy number"?

Response
This sentence has been changed and the appropriate reference added to the manuscript: ‘Complementation-based analyses of CLE mutant strains were performed using pHT315-derived plasmids, which have a copy number of approximately 15 per cell (Arantes & Lereclus 1991).

8) Are you sure you have all upstream regulatory sequences, and if so, why?

Response
Promoter sequences with appropriate sigma factor recognition sequences were identified by sequence analysis so we are confident that the regulatory sequences were captured. Successful complementation analyses indicate also that the cloned ORFs plus upstream sequences were expressed.

9) l 207 - is the fusion protein even expressed- as determined by a Western?

Response
We didn’t check this but given that the upstream regulatory sequence was identical to that used in complementation analyses it seems highly likely that the fusion protein was expressed.

10) Fig. 2, legend - state explicitly that the green fluorescence is due to the fusion protein, as the parental strain shows no fluorescence.

Response
The appropriate sentence in the legend has been modified to read: ‘Green fluorescence is associated with the BMQ_3234-GFP fusion protein and red fluorescence with the membrane-staining FM4-64 dye.’

11) In Fig. 3, give the relevant genotype in the legend so readers don't have to go back to the strain Table.

Response
Strain identifiers and full genotypes have been added to the figure legend.

12 Table 1 - were backgrounds subtracted from values for RFU in Table 1?

Response
Yes. The footnote now reads: 'β-galactosidase values (relative fluorescence units [RFU]) are the average of triplicate measurements, with background values subtracted, conducted with two different spore preparations.'

Reviewer: 2

Comments to the Author
This paper by Al-Riyami et al describes the identification of two apparent spore cortex lytic enzymes that can play minor roles in B. megaterium spore germination. This data can help to explain previously observed differences between this species and some other Bacilli, though, as the authors note, it leaves unresolved, and even possibly complicates the question of how YpeB (and possibly the N-terminal domain of SleB), which does not have lytic activity, facilitates the activity of other enzymes that are not even expected to reside in the same location in the spore.
The methods are properly applied, the data are clearly presented, and the conclusions are mostly justified. My concerns are with one major point of the complementation studies and with under-presentation of bioinformatic analyses of the loci.

1. The inclusion of additional information regarding the new loci under study would help the reader (and authors) to interpret the data.
a. Table 2: Do any of these proteins have predicted signal sequences? (For comparison, SleB does and CwlJ does not.) This could also help with analysis of the GFP-fusion study (see below)

Response
The four SleB homologues have predicted signal sequences whereas the CwlJ homologue doesn't. The manuscript has been updated to include the following sentences: 'Sequence analysis revealed possible sigma G (σ^G) consensus sequences and predicted signal peptide sequences for all four SleB homologues, which is consistent with expression in the forespore during sporulation. In contrast, the BMQ_3234 ORF is preceded by a potential sigma E (σ^E) recognition sequence and lacks a recognisable signal peptide, which is consistent with expression in the mother cell during sporulation.' Table 2 has also been updated to include signal sequence information.

b. Are any of these loci in predicted operons? If so, it greatly complicates the complementation studies. This might also inform the surprising inability to create deletion mutations in these genes and the odd, perhaps obligatory, loss of GerU in the mutant strains.

Response
Neither BMQ_2391 nor BMQ_3234 are predicted to reside in operons. We're not sure why it proved difficult to create deletion mutants in these genes, although we've experienced similar problems with several other spore associated genes. Loss of the large plasmid that encodes GerU is also very common during mutant construction. Ultimately, an improved mutagenesis method for B. megaterium is required.

2. Lines 206-210: The fact that a GFP fusion to BMQ_3234 works and a fusion to BMQ_2391 does not might be related to the localization of the protein. If BMQ_3234 is expressed in the mother cell and localizes to the spore coats, as for CwlJ, the fusion protein would not have to cross a membrane, similarly to CwlJ, for which a GFP fusion is functional. If BMQ_2391 has to cross a membrane for incorporation into the mature spore, then GFP will not work, as for SleB. Do these proteins have predicted signal sequences?

Response

That's a good point. BMQ_2391 almost certainly has a signal sequence (as detailed above), meaning that the N-terminal fusion was unlikely to ever work! The manuscript has been modified to read: 'In contrast, no fluorescence was observed during sporulation or in mature spores for the BMQ_2391-GFP protein, perhaps due to aberrant protein folding or problems associated with the GFP fusion protein translocating the inner membrane (data not shown). A strain designed to express an N-terminal GFP fusion protein also failed to show any detectable fluorescence during sporulation or in mature spores.'

3. It is especially hard to explain how complementation with YpeB works along with BMQ_3234, which is apparently in the spore coat, while YpeB is expected to be associated with the inner spore membrane. This raises the question of whether YpeB (and SleBN) are actually needed for this complementation. The complementation is done with a "low copy" plasmid, but what is the actual copy number, and more importantly, what is the expression level of BMQ-2391 and BMQ_3234 from this plasmid? It is possible that simply several-fold overexpression of these gene products is sufficient to provide the cortex lysis needed to compete the observed slow germination. The presence of SleBN and YpeB on the complementation plasmids might not be required at all! To properly address this, complementation plasmids carrying the new novel genes alone should be tested.

Response

Two new strains were constructed to test this possibility (BR115 and BR116, added to Table 1). The viability of these spores was not appreciably different to Δ*sleB* Δ*cwlJ* spores indicating that YpeB is required for efficient cortex hydrolysis in the Δ*sleB* Δ*cwlJ* background as opposed to over-expression of CLEs (the plasmid copy number is approximately 15 per cell). How YpeB influences CLEs that are synthesised in the mother cell and deposited in the spore coat is a mystery, although the Moir lab provided evidence via antibody labelling that YpeB has a dual location (coat and inner membrane; equally, our own unpublished super resolution analyses indicate inner membrane only). A sentence has been added to the manuscript: 'In contrast, the viability of triple mutant spores complemented with either BMQ_2391 or BMQ_3234 was comparable to spores of the respective non-complemented strains, indicating that YpeB is essential for cortical depolymerisation in the *sleB cwlJ* background.'

Novel cortex lytic enzymes in *Bacillus megaterium* QM B1551 spores

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One sentence summary: Proteins encoded at BMQ_2391 and BMQ_3234 encode novel cortex lytic enzymes that permit cortex hydrolysis in *Bacillus megaterium* spores deficient in the major peptidoglycan lysins SleB and CwlJ.

Abstract

Present models for spore germination in *Bacillus* species include a requirement for either the SleB or CwlJ cortex lytic enzymes to ~~initiate and~~ efficiently depolymerise the spore cortex. Previous work has demonstrated that *B. megaterium* spores may differ to other species in this regard, since *sleB cwlJ* null mutant spores complemented with ~~defined~~the gene in *trans* for the non-peptidoglycan lysin ~~genes in *trans*~~YpeB can efficiently degrade the cortex. Here we identify two novel cortex lytic enzymes, encoded at the BMQ_2391 and BMQ_3234 loci, that are essential for cortex hydrolysis in the absence of SleB and CwlJ. Ellipsoid localisation microscopy places the BMQ_3234 protein within the inner-spore coat, a region of the spore that is populated by other cortex lytic enzymes. The findings reinforce the idea that there is a degree of variation in mechanisms of cortex hydrolysis across the *Bacillales* ~~are not entirely consistent and raise~~, raising potential implications for environmental decontamination strategies based upon targeted inactivation of components of the spore germination apparatus.

Keywords: *Bacillus*, spore germination, cortex lytic enzyme, peptidoglycan lysin

Introduction

Bacteria of the *Bacillales* and *Clostridiales* form metabolically dormant endospores (spores) in response to nutrient limitation. The cellular properties of spores, which ~~encompass~~include several unique morphological and physiological features, means that they are equipped to persist in the environment, in a dormant state, until conditions are conducive to support vegetative growth. The latter is indicated by the presence of nutrient molecules, typically amino acids, monosaccharides and nucleosides, which interact with germinant receptor molecules to trigger spore germination (Setlow *et al.* 2017). A major event in spore germination concerns depolymerisation of the spore cortex, a thick layer of structurally unique peptidoglycan that envelops the spore protoplast and which is a major contributory factor to the maintenance of spore dormancy (Imae & Strominger 1976; Popham & Bernhards 2015). In ~~*Bacillus*~~spores of all species~~, and some *Clostridia*~~, examined the cortex is degraded by specialised peptidoglycan lysins, which are referred to as cortex lytic enzymes (CLEs). It is well established that two main CLEs, SleB and CwlJ, can initiate hydrolysis of the cortex during the germination process (Moriyama *et al.* 1996; Ishikawa *et al.* 1998). SleB and CwlJ are semi-redundant enzymes, in the sense that deletion of either gene permits cortex hydrolysis to an extent where at least a proportion of the spores within a population will retain viability on rich culture medium. Deletion of both genes, however, results in spores that are severely compromised in their capacity to depolymerise the cortex resulting in arrested germination and severely reduced colony forming ability (Ishikawa *et al.* 1998; Heffron *et al.* 2009; Setlow *et al.* 2009).

As with other species of *Bacillus* where CLEs have been characterised by mutational analysis, *Bacillus megaterium* spores that are null for *sleB* and *cwlJ* lose absorbance (A600 nm) when suspended in buffer supplemented with germinants (Setlow *et al.* 2009). A reduction in A600 of approximately 15% is indicative of spores that have released calcium dipicolinate (CaDPA) from the spore core but cannot proceed to cortical depolymerisation and subsequent core hydration (for reference, the A600 of wild type spore suspensions decreases by approximately 60% when germinated under similar circumstances). Furthermore, the viability of *B. megaterium sleB cwlJ* spores is reduced by several orders of magnitude compared to the isogenic wild type strain. Unexpectedly, the introduction on a low copy number plasmid of a truncated *sleB* gene, encoding only the N-terminal non-catalytic domain, plus *ypeB*, which resides in the same operon as *sleB*, restores spore viability and absorbance loss to near wild type levels in the *sleB cwlJ* spores

background (Christie *et al.* 2010). Structural analysis of cortical fragments produced during germination of these spores, and in spores where only *ypeB* was complemented, which also germinate efficiently~~, revealed a relative abundance of anhydromuropeptides in the germination milieu.~~ (Ustok *et al.* 2014), revealed a relative abundance of anhydromuropeptides in the germination milieu. These are the products of a class of peptidoglycan lysin referred to as a lytic transglycosylase. Since SleB is a well characterised lytic transglycosylase (Boland *et al.* 2000; Jing *et al.* 2012; Li *et al.* 2012), the obvious conclusion here is that an orthologue of SleB is present in *B. megaterium* spores, which – like SleB - has an undefined requirement for YpeB, and which can initiate cortex depolymerisation in the absence of SleB and CwlJ.

Accordingly, the objective of the current study was to identify and characterise novel CLEs that are functional during the germination of *B. megaterium* QM B1551 spores, and in particular, those that permit efficient cortex hydrolysis in the absence of SleB and CwlJ.

Materials and Methods

Bacterial strains and spore preparation

B. megaterium strains employed in this study, which were all isogenic with the QM B1551 strain, were cultured routinely at 30°C on LB medium supplemented where appropriate with antibiotics (Table 1). Spores were prepared by nutrient exhaustion in supplemented nutrient broth and subsequently purified by repeated rounds of centrifugation and resuspension of spore pellets in deionised ice-cold water (Christie *et al.* 2010). Purified suspensions comprising >99% phase bright spores were stored in deionised water, at an A600 of approximately 50, on ice. *Escherichia coli* Top 10 cells (Thermo Fisher, UK) were used for cloning procedures, plasmid propagation and for heterologous protein expression experiments.

Spore germination and viability assays

Spore germination in liquid medium was assessed in microtitre plates by adding 10 µl of heat-shocked (80°C, 30 min) spores to 190 µl of 5% (wt/vol) beef extract (Oxoid, Ltd., Basingstoke, United Kingdom), preheated to 37°C. The A600 of spore suspensions was 0.4. Plates were sealed with adhesive film to minimize evaporative losses, and then incubated in a Perkin-Elmer EnVision-Xcite multilabel plate reader fitted with a 600 nm photometric filter. Plates were agitated orbitally for 10 seconds prior to absorbance measurements, which were recorded every minute for

90 minutes. Experiments were conducted in triplicate, with at least two or more independent spore preparations. Spore viability was determined by pipetting 10 µl aliquots of serially diluted suspensions of heat-shocked spores (A600 of 1 [10^8 spores/ml]) onto LB agar plates. Plates were incubated for 16 to 24 h prior to colony enumeration.

Molecular biology procedures

Transcriptional analysis from loci of interest was examined by RT-PCR, using RNA purified from sporulating cultures, a QuantiTect Reverse Transcription kit (Qiagen, Manchester, UK) and gene-specific primers designed to amplify approximately 400 bp fragments, essentially as described previously (Ramirez-Peralta *et al.* 2013).

Inactivation of BMQ_2391 and BMQ_3234 in the *B. megaterium sleB cwlJ* background was accomplished by integrating pUCTV2 derived plasmids at the respective loci. Essentially, the first 300 bp of each gene was amplified from *B. megaterium* QM B1551 genomic DNA by PCR. Purified PCR products were ligated with pUCTV2 (digested with EcoRI), and ligation mixtures subsequently used to transform *E. coli* to carbenicillin resistance. Isolated plasmids were introduced to *B. megaterium* protoplasts using standard polyethylene glycol mediated transformation procedures. Tetracycline resistant transformants were then repeatedly streaked on LB agar plates containing 1.25 µg/ml tetracycline at 42°C to permit isolation of clones that had integrated plasmid DNA at the cloned loci, prior to validating by PCR and sequencing. Strains with translational *gfp* fusions to genes of interest were constructed by amplifying entire BMQ_2391 and BMQ_3234 ORFs, minus stop codons, using primers that incorporated XhoI and EcoRI restriction sites at the respective 5' and 3' ends. Purified and digested PCR amplicons were ligated with similarly digested pVLG6 vector to create 3' fusions to *gfp*. The resultant plasmids were purified from *E. coli* and used to transform *B. megaterium* to chloramphenicol resistance. Individual colonies were streaked and incubated at the non-permissive temperature of 42°C in the presence of chloramphenicol, to select for plasmid integrations into the chromosome at cloned loci. Procedures designed to create spores with transcriptional fusions between putative CLE promoter sequences and *lacZ* were constructed using plasmid pNFd13, and resultant measurements of β-galactosidase activity associated with disrupted spores, were as described previously (Gupta *et al.* 2013).

Complementation-based analyses of CLE mutant strains were performed using ~~low copy~~

~~number pHT315-derived plasmids.~~pHT315-derived plasmids, which have a copy number of approximately 15 per cell (Arantes & Lereclus 1991). The existing pHT-*sleB*^{N} *ypeB* plasmid (Christie *et al.* 2010) was modified to additionally incorporate ORFs and upstream regulatory sequences for either BMQ_2391 or BMQ_3234 proteins. This was achieved using EcoRI restricted pHT-*sleB*^{N} *ypeB* and BMQ_2391 or BMQ_3234 PCR amplicons designed to facilitate cloning via the Klenow Assembly Method (Ghosh *et al.* 2018). Similar procedures were used to introduce BMQ_2391 or BMQ_3234 ORFs and regulatory sequences into pHT315. The *B. megaterium sleB cwlJ BMQ_2391* and *B. megaterium sleB cwlJ BMQ_3234* strains were subsequently transformed, using the appropriate ~~plasmid~~plasmids, to erythromycin and lincomycin resistance.

Ellipsoid localisation microscopy

The quantitative fluorescence ELM technique was used to measure the location of GFP fusion proteins in mature spores (Manetsberger *et al.* 2015; Manton *et al.* 2018). Briefly, several independent fields of GFP labelled spores were imaged using an Olympus BX53 microscope fitted with a 100X 1.30 NA oil objective lens, with illumination from a mercury lamp, filters for GFP fluorescence, and a Retiga 2000R CCD camera. Automated image segmentation was used to identify single spores, and the image of each candidate was used to fit the parameters of a model that describes the image of a spheroidal fluorescent shell. For *B. megaterium*, an equation describing the image of a spherical fluorescent layer was fitted to the image data. A filter was applied to exclude fits from overlapping spores and fragments of debris. The average radius parameter fitted to the spores provides an estimate of the midpoint radial position of the GFP fusion with respect to the spore centre.

Protein interactions

A bacterial adenylate cyclase two-hybrid (BACTH) system (Euromedex, Souffelweyersheim, France) was used to characterise potential interactions *in vivo* between *B. megaterium* CLEs and related proteins (BMQ_2391, BMQ_3234, CwlJ, SleB, SleL and YpeB). The BACTH system is based upon two complementary adenylate cyclase fragments (T25 and T18) being brought together by interacting fusion partners to form functional enzyme, resulting in cAMP synthesis and activation of a *lacZ* reporter gene. Plasmids used for BACTH assays in this work were prepared by PCR amplifying ORFs encoding CLEs and YpeB from genomic DNA before assembling with

linearised pUT18 and pKT25 plasmids by Klenow assembly. The resulting plasmids, designed for expression of C-terminal fusions between proteins of interest and T25 or T18 adenylate cyclase fragments, were isolated and verified by sequencing. Electrocompetent *E. coli* BTH101 were co-transformed using various combinations of pKT25 and pUT18 derived plasmids and selected on LB agar plates supplemented with 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 1 mM IPTG, and 50 µg/ml carbenicillin and kanamycin, respectively. Cells were cultured for 24-72 h at 30°C and examined for the development of blue coloration, which is indicative of positive interactions between pairs of fusion proteins.

Results

Identifying potential CLE genes

Genes encoding candidate novel CLEs were identified by conducting NCBI Protein BLAST searches using sequences for the C-terminal catalytic domain of SleB and full length CwlJ as probes against the *B. megaterium* QM B1551 genome (GenBank accession numbers CP001983 – CP001900). The searches identified five potential CLEs, four of which showed greater amino acid sequence identity with the catalytic domain of SleB (ranging from 40 – 46 %), whereas BMQ_3234 shared greater sequence identity to CwlJ (49%) (Table 2). Sequence analysis revealed possible sigma G (σ^G) consensus sequences and predicted signal peptide sequences for all four SleB homologues, which is consistent with expression in the forespore during sporulation. In contrast, the BMQ_3234 ORF is preceded by a potential sigma E (σ^E) recognition sequence and lacks a recognisable signal peptide, which is consistent with mother cell expression in the mother cell during sporulation. All five predicted proteins are members of the Hydrolase-2 family (PF07486), which is consistent with a role in peptidoglycan depolymerisation. Two of the putative proteins contain single peptidoglycan-binding LysM domains (BMQ_1284 and BMQ_2145) with BMQ_3195 predicted to have two N-terminal LysM domains. LysM domains are present in established spore CLEs, including SleL (Ustok *et al.* ~~2015b~~2015). In contrast, neither BMQ_2391 nor BMQ_3234 appear to contain defined substrate binding domains, which is analogous to the domain architecture of CwlJ. Pairwise sequence alignments indicate that SleB's single catalytic residue, E208, is present and conserved in the primary sequence of all five putative CLEs (Table 2).

Transcriptional analysis of candidate CLE genes

In order to ascertain whether the various candidate CLE genes were transcribed during sporulation, RT-PCR was conducted on cDNA derived from *B. megaterium* cultures sampled immediately prior to entry to stationary phase and at hourly intervals thereafter (Figure 1). These analyses indicate that two of the candidate CLEs - BMQ_1284 and BMQ_2145 – are transcribed at very low levels based on the low abundance of RT-PCR products. BMQ_2391 is transcribed from the early to mid-stages of sporulation, with a pattern of expression that most closely matches that of *sleB*. In contrast, BMQ_3195 transcripts are most abundant from the mid to latter stages of

sporulation, whereas BMQ_3234 appears to be transcribed strongly throughout. The latter pattern of transcription matches that of *cwlJ*. Further transcriptional analyses of candidate and established CLE promoters were achieved by placing the *lacZ* gene under control of putative promoter sequences for the various genes. The outputs from β-galactosidase assays conducted with supernatants from disrupted spores of the various *lacZ* reporter strains are broadly in line with results from the aforementioned RT-PCR assays i.e. where BMQ_1284 and BMQ_2145 transcriptional fusions are associated with little β-galactosidase activity, while BMQ_2391 and BMQ_3234 show the highest levels of expression, albeit less than evident for *sleB* and *cwlJ* transcriptional fusions (Table 1).

Localisation of BMQ_2391 and BMQ_3234 GFP fusion proteins

Proteins encoded at the BMQ_2391and BMQ_3234 loci were selected for further analysis since they appeared to be expressed at the highest levels of the candidate CLEs. The sub-cellular localisation of both proteins was investigated during sporulation and in mature spores by creating constructs designed to express C-terminal GFP fusion proteins. Fluorescence microscopy revealed that the BMQ_3234-GFP protein is expressed during sporulation in the mother cell compartment and is deposited to form a ring of fluorescence around the developing forespore (Figure 2). A strong fluorescence signal was retained in mature spores (Figure 2). The average radial location of the BMQ_3234-GFP protein, as inferred from ellipsoid localisation analyses, was 521 nm ±9 nm in mature spores (where ±9 nm is the standard deviation of radial locations found in repeated measurements and where 865 spores were analysed with a residual fitting error of 8.4%). This places the location of BMQ_3234 to the inner spore coat, within the same vicinity as SleL (525 nm ±11 nm; 362 spores analysed with a residual fitting error of 6.5%), and presumably CwlJ (Setlow *et al.* 2017; Ghosh *et al.* 2018). In contrast, no fluorescence was observed during sporulation or in mature spores for the BMQ_2391-GFP protein, ~~presumably~~perhaps due to aberrant protein folding or ~~mis-localisation of~~problems associated with the GFP fusion protein translocating the inner membrane (data not shown). ~~Consequently, another~~A strain ~~was constructed, in this case~~ designed to express an N-terminal GFP fusion protein~~. Again,~~ also failed to show any detectable fluorescence ~~signal was not detected throughout~~during sporulation or in mature spores.

Germination of BMQ_2391 and BMQ_3234 null mutant spores

To investigate whether the BMQ_2391 and BMQ_3234 proteins have a role in germination, particularly in the absence of the major CLEs, mutant strains lacking either of these enzymes were constructed in the *B. megaterium sleB cwlJ* double mutant background. Despite repeated attempts we failed to isolate mutants that had undergone allelic exchange with truncated and disrupted gene variants at either locus. Instead, strains that had undergone single recombination events, integrating pUCTV2 derived plasmids at the target loci and thereby separating the respective ORFs from their promoter sequences, were isolated. Copies of *sleB*^{N} and *ypeB* under control of the native promoter sequence were introduced to these strains on a low copy number pHT315 derived plasmid, and the resultant strains sporulated by nutrient starvation. Both strains were found to have excised the pBM600 plasmid during mutagenesis. This plasmid encodes the GerU-germinant receptor, hence the resultant spores were heat shocked and germinated in beef extract medium, which promotes an efficient germinative response in the absence of GerU (Gupta *et al.* 2013). Germination assays conducted with spores suspended in beef extract revealed that both triple mutant strains - *sleB cwlJ BMQ_2391* and *sleB cwlJ BMQ_3234* - complemented with plasmid bourne *sleB*^{N} and *ypeB*, had major germination defects, with the A600 decreasing by a comparable amount (~15 %) to *sleB cwlJ* spores (Figure 3). This is indicative of CaDPA efflux but limited, if any, cortex depolymerisation. The viability of both triple mutant strains was also severely compromised compared to wild type and *sleB cwlJ* spores complemented with *sleB*^{N} *ypeB*, again being comparable to *sleB cwlJ* spores lacking plasmid borne *sleB*^{N} *ypeB* (Table 1). In contrast, the viability of triple mutant strains complemented with plasmid borne copies of *sleB*^{N} *ypeB* and *BMQ_2391* or *BMQ_3234*, as appropriate, was restored to approximately half of that observed in *sleB cwlJ* pHT-*sleB*^{N} *ypeB* spores (35-40 % versus 80 %). Similarly, complemented spores show a reduction in A600 of ~50 % within 40 minutes in beef extract, which although not as efficient as *sleB cwlJ* pHT-*sleB*^{N} *ypeB* spores, is indicative of significant cortical depolymerisation (Figure 3). In contrast, the viability of triple mutant spores complemented with either BMQ_2391 or BMQ_3234 was comparable to spores of the respective non-complemented strains, indicating that YpeB is essential for cortical depolymerisation in the *sleB cwlJ* background.

Interactions between CLEs

Results with null mutant spores reported in this work, and in previous studies (Bernhards & Popham 2014; Ustok *et al.* 2014), indicate that direct physical interactions between CLEs and or CLEs and the YpeB protein may be required to facilitate efficient cortex hydrolysis during germination. The *E. coli* based BACTH system was employed in the current study to investigate potential interactions between the two novel CLEs identified in this work – BMQ_2391 and BMQ_3234 – with each other and with SleB, CwlJ, SleL and YpeB. Potential interactions between (i) SleB with YpeB, SleL and CwlJ, (ii) CwlJ with YpeB and SleL, and (iii) SleL with YpeB, were also examined. Blue coloration of co-transformant *E. coli* colonies was not observed in any of the combinations tested, with the exception of the positive control, indicating that none of the spore proteins of interest interact productively in this system (Figure 4).

Heterologous expression of BMQ_2391 and BMQ_3234

Several attempts were made to express recombinant versions of the BMQ_2391 and BMQ_3234 proteins with a view to characterising the hydrolytic bond specificity of each protein. Hosts for heterologous expression included *E. coli*, *Lactococcus lactis* and *B. subtilis*, with several variant proteins – including truncated forms, GFP, and maltose binding protein (solubility enhancer) fusions – being examined over the course of this work. Unfortunately, levels of expression commensurate with biochemical analyses were not achieved in any of the systems employed (data not shown).

Discussion

Initiation of cortex hydrolysis in current models of *Bacillus* spore germination requires the activity of either of the semi redundant CLEs SleB or CwlJ. Efficient degradation of the cortex is subsequently facilitated by enzymes such as SleL, whose function appears to be associated with further hydrolysis of large peptidoglycan fragments generated by SleB and or CwlJ (Chen *et al.* 2000; Lambert & Popham 2008; Ustok *et al.* 2015). While this sequence of events probably occurs in wild type spores of all species of *Bacillus*, mutagenesis analyses conducted with *B. megaterium sleB cwlJ* spores have revealed that this species can circumvent the requirement for either of SleB or CwlJ . Previous work revealed that *B. megaterium sleB cwlJ* spores could degrade the cortex with an efficiency comparable to wild type spores when complemented with a plasmid-borne copy of $sleB^N$, which encodes the non catalytic domain of SleB, plus the adjacent *ypeB* gene (Christie

et al. 2010). Indeed, complementation with *ypeB* alone restored efficient cortex hydrolysis to *sleB cwlJ* spores (Ustok *et al.* 2014). The precise function of YpeB has not been determined, although it is not a peptidoglycan lysin, and instead appears to have a role in localising – and perhaps regulating the activity - of SleB in the spore (Boland *et al.* 2000; Bernhards & Popham 2014; Ustok *et al.* 2015).

Another study in *B. megaterium* extended these findings and attempted to dissect the molecular mechanisms that support efficient cortex hydrolysis in *sleB cwlJ* spores (Ustok *et al.* 2014). In this case, a triple mutant (*sleB cwlJ sleL*) strain complemented with $sleB^N$ plus *ypeB* showed a severe germination defect. Based on this result, the study concluded that *ypeB* and *sleL* are both essential for the initiation of cortex hydrolysis in *B. megaterium sleB cwlJ* spores. The requirement for *sleL* in the *sleB cwlJ* background was unexpected since, as noted above, SleL is regarded as a cortical fragment lytic enzyme. Similarly, muropeptide analyses of germination exudates from *sleB cwlJ* spores complemented with *ypeB* clearly indicate the presence of lytic transglycosylase activity during germination, whereas SleL exhibits N-acetylglucosaminidase activity (Lambert & Popham 2008; Ustok *et al.* 2014).

With this context in mind, the main purpose of the current study was to identify cortex lytic enzymes that are functional in *B. megaterium sleB cwlJ* spores complemented with plasmid borne *ypeB*. Progress in this regard was achieved via bioinformatic analyses, which identified five candidate CLE loci. Subsequent transcriptional analyses, comprising RT-PCR and β-galactoside reporter assays, were used to narrow candidates for further characterisation to proteins encoded at the BMQ_2391 and BMQ_3234 loci (moderate transcription, relative to *sleB* and *cwlJ*, was associated with BMQ_3195, although this protein was not characterised further).

Mutagenesis analyses in this work were compromised to a certain extent in that we could not isolate null mutant strains that had undergone allelic exchange with truncated and disrupted versions of *BMQ_2391* or *BMQ_3234* in the *sleB cwlJ* background. However, strains in which *BMQ_2391* or *BMQ_3234* were disrupted by integrative plasmids, effectively separating the promoter and first 300 nucleotides of the respective genes from the remainder of the coding sequences, were isolated, albeit at the expense of the GerU-encoding pBM600 plasmid. Loss of the GerU germinant receptor can be circumvented by germinating *gerU* spores in beef extract, components of which trigger efficient germination responses via alternative germinant receptors in *B. megaterium* (Gupta *et al.* 2013). In both cases, germination of triple mutant spores – *sleB*

cwlJ BMQ_2391 and *sleB cwlJ BMQ_3234* – complemented with plasmid borne *sleB*^{N} and *ypeB*, was comparable in terms of absorbance loss to non *sleB*^{N} *ypeB* complemented triple mutant spores. The observed reduction in A600 of approximately 15% is indicative of spores that have released CaDPA and various ions from the spore core but which have failed to depolymerise cortical peptidoglycan. Viability of the *sleB*^{N} *ypeB* complemented triple mutant spores was similarly comparable to non-complemented spores, with colony forming ability on LB medium being reduced by more than five logs compared to isogenic wild type spores, indicating again that the cortex has not been degraded. In contrast, complementation in trans with *BMQ_2391* or *BMQ_3234* in *sleB*^{N} *ypeB* complemented triple mutant spores restored viability and absorbance loss to approximately 40% of wild type levels.

Outputs from the present study support the hypothesis that *BMQ_2391* and *BMQ_3234* encode novel CLEs that are not only active during germination of *B. megaterium* spores, but which also confer further redundancy within the cortical depolymerisation system of this species (and perhaps this species alone since *sleB*^{N} *ypeB* in trans failed to restore cortex hydrolysis to *B. subtilis sleB cwlJ* spores (Li *et al.* 2013)). In some regards the requirement for three separate enzymes - SleL, BMQ_2391 and BMQ_3234 – in tandem with YpeB, for efficient spore germination in *B. megaterium sleB cwlJ* spores, is difficult to reconcile. One possibility is that the combined activity of each enzyme – which may be infrequent cutters – is required for cortex depolymerisation to a degree that is commensurate with germination. It's possible also that at least some of these proteins physically interact to ensure correct localisation and or function in the spore. Ellipsoid localisation microscopy analysis places SleL and BMQ_3234 within the same inner coat location within the spore, which would facilitate physical interactions. However, where examined - bacterial two-hybrid assays in this work and pull-down assays conducted previously (Li *et al.* 2013; Ustok *et al.* 2014) – have failed to provide any evidence of interactions between CLEs. ~~Accordingly, further~~Further insight to the nature of the inter-dependency between CLEs, and how YpeB may contribute to this, are objectives for continuing work in this area. Finally, targeted inactivation of CLEs may provide a novel strategy for spore decontamination in a number of sectors. A potential implication arising from the present study is that enzymes in addition to the major CLEs may have to be inhibited, at least in some species, for efficient spore inactivation.

Acknowledgements

BAR was the recipient of a scholarship from the Sultanate of Oman government. AG was the recipient of a Cambridge Nehru Scholarship and received support from the Raymond and Beverly Sackler Foundation. Contributions to this work by Fatma Isik Ustok, Srishti Gupta, Ke Xu Zhou and Akshay Deshmukh are acknowledged.

Conflicts of interest

None declared

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