

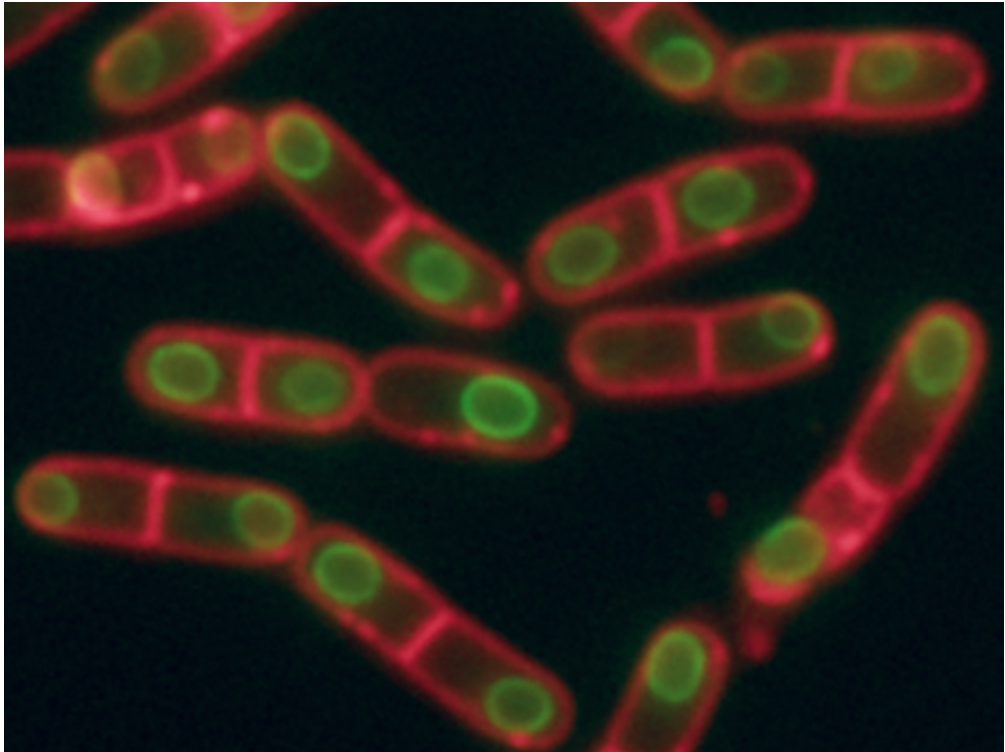
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Novel cortex lytic enzymes in *Bacillus megaterium* QM B1551 spores

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3 1 **Novel cortex lytic enzymes in *Bacillus megaterium* QM B1551 spores**
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26 11 One sentence summary: Proteins encoded at BMQ_2391 and BMQ_3234 encode novel cortex
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28 12 lytic enzymes that permit cortex hydrolysis in *Bacillus megaterium* spores deficient in the major
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30 13 peptidoglycan lysins SleB and CwlJ.
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3 15 **Abstract**
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6 16 Present models for spore germination in *Bacillus* species include a requirement for either the SleB
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8 17 or CwlJ cortex lytic enzymes to efficiently depolymerise the spore cortex. Previous work has
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10 18 demonstrated that *B. megaterium* spores may differ to other species in this regard, since *sleB cwlJ*
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12 19 null mutant spores complemented with the gene in *trans* for the non-peptidoglycan lysin YpeB can
13
14 20 efficiently degrade the cortex. Here we identify two novel cortex lytic enzymes, encoded at the
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16 21 BMQ_2391 and BMQ_3234 loci, that are essential for cortex hydrolysis in the absence of SleB
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18 22 and CwlJ. Ellipsoid localisation microscopy places the BMQ_3234 protein within the inner-spore
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20 23 coat, a region of the spore that is populated by other cortex lytic enzymes. The findings reinforce
21
22 24 the idea that there is a degree of variation in mechanisms of cortex hydrolysis across the *Bacillales*,
23
24 25 raising potential implications for environmental decontamination strategies based upon targeted
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26 26 inactivation of components of the spore germination apparatus.

26 27 Keywords: *Bacillus*, spore germination, cortex lytic enzyme, peptidoglycan lysin
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29 Introduction

30 Bacteria of the *Bacillales* and *Clostridiales* form metabolically dormant endospores (spores) in
31 response to nutrient limitation. The cellular properties of spores, which include several unique
32 morphological and physiological features, means that they are equipped to persist in the
33 environment, in a dormant state, until conditions are conducive to support vegetative growth. The
34 latter is indicated by the presence of nutrient molecules, typically amino acids, monosaccharides
35 and nucleosides, which interact with germinant receptor molecules to trigger spore germination
36 (Setlow *et al.* 2017). A major event in spore germination concerns depolymerisation of the spore
37 cortex, a thick layer of structurally unique peptidoglycan that envelops the spore protoplast and
38 which is a major contributory factor to the maintenance of spore dormancy (Imae & Strominger
39 1976; Popham & Bernhards 2015). In spores of all species examined the cortex is degraded by
40 specialised peptidoglycan lysins, which are referred to as cortex lytic enzymes (CLEs). It is well
41 established that two main CLEs, SleB and CwlJ, can initiate hydrolysis of the cortex during the
42 germination process (Moriyama *et al.* 1996; Ishikawa *et al.* 1998). SleB and CwlJ are semi-
43 redundant enzymes, in the sense that deletion of either gene permits cortex hydrolysis to an extent
44 where at least a proportion of the spores within a population will retain viability on rich culture
45 medium. Deletion of both genes, however, results in spores that are severely compromised in their
46 capacity to depolymerise the cortex resulting in arrested germination and severely reduced colony
47 forming ability (Ishikawa *et al.* 1998; Heffron *et al.* 2009; Setlow *et al.* 2009).

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

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3 60 germination of these spores, and in spores where only *ypeB* was complemented, which also
4 61 germinate efficiently (Ustok *et al.* 2014), revealed a relative abundance of anhydromuropeptides
5 62 in the germination milieu. These are the products of a class of peptidoglycan lysin referred to as a
6 63 lytic transglycosylase. Since SleB is a well characterised lytic transglycosylase (Boland *et al.* 2000;
7 64 Jing *et al.* 2012; Li *et al.* 2012), the obvious conclusion here is that an orthologue of SleB is present
8 65 in *B. megaterium* spores, which – like SleB - has an undefined requirement for YpeB, and which
9 66 can initiate cortex depolymerisation in the absence of SleB and CwlJ.

15 67 Accordingly, the objective of the current study was to identify and characterise novel CLEs
16 68 that are functional during the germination of *B. megaterium* QM B1551 spores, and in particular,
17 69 those that permit efficient cortex hydrolysis in the absence of SleB and CwlJ.
20 70

22 71 **Materials and Methods**

24 72 **Bacterial strains and spore preparation**

25 73 *B. megaterium* strains employed in this study, which were all isogenic with the QM B1551 strain,
26 74 were cultured routinely at 30°C on LB medium supplemented where appropriate with antibiotics
27 75 (Table 1). Spores were prepared by nutrient exhaustion in supplemented nutrient broth and
28 76 subsequently purified by repeated rounds of centrifugation and resuspension of spore pellets in
29 77 deionised ice-cold water (Christie *et al.* 2010). Purified suspensions comprising >99% phase bright
30 78 spores were stored in deionised water, at an A600 of approximately 50, on ice. *Escherichia coli*
31 79 Top 10 cells (Thermo Fisher, UK) were used for cloning procedures, plasmid propagation and for
32 80 heterologous protein expression experiments.
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41 82 **Spore germination and viability assays**

42 83 Spore germination in liquid medium was assessed in microtitre plates by adding 10 µl of heat-
43 84 shocked (80°C, 30 min) spores to 190 µl of 5% (wt/vol) beef extract (Oxoid, Ltd., Basingstoke,
44 85 United Kingdom), preheated to 37°C. The A600 of spore suspensions was 0.4. Plates were sealed
45 86 with adhesive film to minimize evaporative losses, and then incubated in a Perkin-Elmer
46 87 EnVision-Xcite multilabel plate reader fitted with a 600 nm photometric filter. Plates were agitated
47 88 orbitally for 10 seconds prior to absorbance measurements, which were recorded every minute for
48 89 90 minutes. Experiments were conducted in triplicate, with at least two or more independent spore
49 90 preparations. Spore viability was determined by pipetting 10 µl aliquots of serially diluted
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3 91 suspensions of heat-shocked spores (A600 of 1×10^8 spores/ml) onto LB agar plates. Plates were
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5 92 incubated for 16 to 24 h prior to colony enumeration.
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8 94 **Molecular biology procedures**

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10 95 Transcriptional analysis from loci of interest was examined by RT-PCR, using RNA purified from
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12 96 sporulating cultures, a QuantiTect Reverse Transcription kit (Qiagen, Manchester, UK) and gene-
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14 97 specific primers designed to amplify approximately 400 bp fragments, essentially as described
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16 98 previously (Ramirez-Peralta *et al.* 2013).

17 99 Inactivation of BMQ_2391 and BMQ_3234 in the *B. megaterium sleB cwlJ* background
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19 100 was accomplished by integrating pUCTV2 derived plasmids at the respective loci. Essentially, the
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21 101 first 300 bp of each gene was amplified from *B. megaterium* QM B1551 genomic DNA by PCR.
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23 102 Purified PCR products were ligated with pUCTV2 (digested with EcoRI), and ligation mixtures
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25 103 subsequently used to transform *E. coli* to carbenicillin resistance. Isolated plasmids were
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27 104 introduced to *B. megaterium* protoplasts using standard polyethylene glycol mediated
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29 105 transformation procedures. Tetracycline resistant transformants were then repeatedly streaked on
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31 106 LB agar plates containing 1.25 $\mu\text{g/ml}$ tetracycline at 42°C to permit isolation of clones that had
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33 107 integrated plasmid DNA at the cloned loci, prior to validating by PCR and sequencing. Strains
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35 108 with translational *gfp* fusions to genes of interest were constructed by amplifying entire
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37 109 BMQ_2391 and BMQ_3234 ORFs, minus stop codons, using primers that incorporated XhoI and
38
39 110 EcoRI restriction sites at the respective 5' and 3' ends. Purified and digested PCR amplicons were
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41 111 ligated with similarly digested pVLG6 vector to create 3' fusions to *gfp*. The resultant plasmids
42
43 112 were purified from *E. coli* and used to transform *B. megaterium* to chloramphenicol resistance.
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45 113 Individual colonies were streaked and incubated at the non-permissive temperature of 42°C in the
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47 114 presence of chloramphenicol, to select for plasmid integrations into the chromosome at cloned
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49 115 loci. Procedures designed to create spores with transcriptional fusions between putative CLE
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51 116 promoter sequences and *lacZ* were constructed using plasmid pNFd13, and resultant
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53 117 measurements of β -galactosidase activity associated with disrupted spores, were as described
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55 118 previously (Gupta *et al.* 2013).

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57 119 Complementation-based analyses of CLE mutant strains were performed using pHT315-
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59 120 derived plasmids, which have a copy number of approximately 15 per cell (Arantes & Lereclus
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121 1991). The existing pHT-*sleB^N ypeB* plasmid (Christie *et al.* 2010) was modified to additionally

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3 122 incorporate ORFs and upstream regulatory sequences for either BMQ_2391 or BMQ_3234
4 123 proteins. This was achieved using EcoRI restricted pHT-*sleB^N* *ypeB* and BMQ_2391 or
5 124 BMQ_3234 PCR amplicons designed to facilitate cloning via the Klenow Assembly Method
6 125 (Ghosh *et al.* 2018). Similar procedures were used to introduce BMQ_2391 or BMQ_3234 ORFs
7 126 and regulatory sequences into pHT315. The *B. megaterium sleB cwlJ BMQ_2391* and *B.*
8 127 *megaterium sleB cwlJ BMQ_3234* strains were subsequently transformed, using the appropriate
9 128 plasmids, to erythromycin and lincomycin resistance.
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17 130 **Ellipsoid localisation microscopy**

18 131 The quantitative fluorescence ELM technique was used to measure the location of GFP fusion
19 132 proteins in mature spores (Manetsberger *et al.* 2015; Manton *et al.* 2018). Briefly, several
20 133 independent fields of GFP labelled spores were imaged using an Olympus BX53 microscope fitted
21 134 with a 100X 1.30 NA oil objective lens, with illumination from a mercury lamp, filters for GFP
22 135 fluorescence, and a Retiga 2000R CCD camera. Automated image segmentation was used to
23 136 identify single spores, and the image of each candidate was used to fit the parameters of a model
24 137 that describes the image of a spheroidal fluorescent shell. For *B. megaterium*, an equation
25 138 describing the image of a spherical fluorescent layer was fitted to the image data. A filter was
26 139 applied to exclude fits from overlapping spores and fragments of debris. The average radius
27 140 parameter fitted to the spores provides an estimate of the midpoint radial position of the GFP fusion
28 141 with respect to the spore centre.
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39 143 **Protein interactions**

40 144 A bacterial adenylate cyclase two-hybrid (BACTH) system (Euromedex, Souffelweyersheim,
41 145 France) was used to characterise potential interactions *in vivo* between *B. megaterium* CLEs and
42 146 related proteins (BMQ_2391, BMQ_3234, CwlJ, SleB, SleL and YpeB). The BACTH system is
43 147 based upon two complementary adenylate cyclase fragments (T25 and T18) being brought together
44 148 by interacting fusion partners to form functional enzyme, resulting in cAMP synthesis and
45 149 activation of a *lacZ* reporter gene. Plasmids used for BACTH assays in this work were prepared
46 150 by PCR amplifying ORFs encoding CLEs and YpeB from genomic DNA before assembling with
47 151 linearised pUT18 and pKT25 plasmids by Klenow assembly. The resulting plasmids, designed for
48 152 expression of C-terminal fusions between proteins of interest and T25 or T18 adenylate cyclase
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3 153 fragments, were isolated and verified by sequencing. Electrocompetent *E. coli* BTH101 were co-
4 154 transformed using various combinations of pKT25 and pUT18 derived plasmids and selected on
5 155 LB agar plates supplemented with 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-
6 156 galactopyranoside), 1 mM IPTG, and 50 µg/ml carbenicillin and kanamycin, respectively. Cells
7 157 were cultured for 24-72 h at 30°C and examined for the development of blue coloration, which is
8 158 indicative of positive interactions between pairs of fusion proteins.
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160 **Results**

161 **Identifying potential CLE genes**

162 Genes encoding candidate novel CLEs were identified by conducting NCBI Protein BLAST
163 searches using sequences for the C-terminal catalytic domain of SleB and full length CwlJ as
164 probes against the *B. megaterium* QM B1551 genome (GenBank accession numbers CP001983 –
165 CP001900). The searches identified five potential CLEs, four of which showed greater amino acid
166 sequence identity with the catalytic domain of SleB (ranging from 40 – 46 %), whereas BMQ_3234
167 shared greater sequence identity to CwlJ (49%) (Table 2). Sequence analysis revealed possible
168 sigma G (σ^G) consensus sequences and predicted signal peptide sequences for all four SleB
169 homologues, which is consistent with expression in the forespore during sporulation. In contrast,
170 the BMQ_3234 ORF is preceded by a potential sigma E (σ^E) recognition sequence and lacks a
171 recognisable signal peptide, which is consistent with mother cell expression in the mother cell
172 during sporulation. All five predicted proteins are members of the Hydrolase-2 family (PF07486),
173 which is consistent with a role in peptidoglycan depolymerisation. Two of the putative proteins
174 contain single peptidoglycan-binding LysM domains (BMQ_1284 and BMQ_2145) with
175 BMQ_3195 predicted to have two N-terminal LysM domains. LysM domains are present in
176 established spore CLEs, including SleL (Ustok *et al.* 2015). In contrast, neither BMQ_2391 nor
177 BMQ_3234 appear to contain defined substrate binding domains, which is analogous to the
178 domain architecture of CwlJ. Pairwise sequence alignments indicate that SleB's single catalytic
179 residue, E208, is present and conserved in the primary sequence of all five putative CLEs (Table
180 2).

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182 **Transcriptional analysis of candidate CLE genes**

183 In order to ascertain whether the various candidate CLE genes were transcribed during sporulation,
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3 184 RT-PCR was conducted on cDNA derived from *B. megaterium* cultures sampled immediately
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5 185 prior to entry to stationary phase and at hourly intervals thereafter (Figure 1). These analyses
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7 186 indicate that two of the candidate CLEs - BMQ_1284 and BMQ_2145 – are transcribed at very
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9 187 low levels based on the low abundance of RT-PCR products. BMQ_2391 is transcribed from the
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11 188 early to mid-stages of sporulation, with a pattern of expression that most closely matches that of
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13 189 *sleB*. In contrast, BMQ_3195 transcripts are most abundant from the mid to latter stages of
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15 190 sporulation, whereas BMQ_3234 appears to be transcribed strongly throughout. The latter pattern
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17 191 of transcription matches that of *cwlJ*. Further transcriptional analyses of candidate and established
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19 192 CLE promoters were achieved by placing the *lacZ* gene under control of putative promoter
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21 193 sequences for the various genes. The outputs from β -galactosidase assays conducted with
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23 194 supernatants from disrupted spores of the various *lacZ* reporter strains are broadly in line with
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25 195 results from the aforementioned RT-PCR assays i.e. where BMQ_1284 and BMQ_2145
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27 196 transcriptional fusions are associated with little β -galactosidase activity, while BMQ_2391 and
28
29 197 BMQ_3234 show the highest levels of expression, albeit less than evident for *sleB* and *cwlJ*
30
31 198 transcriptional fusions (Table 1).

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200 **Localisation of BMQ_2391 and BMQ_3234 GFP fusion proteins**

201 Proteins encoded at the BMQ_2391 and BMQ_3234 loci were selected for further analysis since
202 they appeared to be expressed at the highest levels of the candidate CLEs. The sub-cellular
203 localisation of both proteins was investigated during sporulation and in mature spores by creating
204 constructs designed to express C-terminal GFP fusion proteins. Fluorescence microscopy revealed
205 that the BMQ_3234-GFP protein is expressed during sporulation in the mother cell compartment
206 and is deposited to form a ring of fluorescence around the developing forespore (Figure 2). A
207 strong fluorescence signal was retained in mature spores (Figure 2). The average radial location of
208 the BMQ_3234-GFP protein, as inferred from ellipsoid localisation analyses, was $521 \text{ nm} \pm 9 \text{ nm}$
209 in mature spores (where $\pm 9 \text{ nm}$ is the standard deviation of radial locations found in repeated
210 measurements and where 865 spores were analysed with a residual fitting error of 8.4%). This
211 places the location of BMQ_3234 to the inner spore coat, within the same vicinity as SleL (525
212 $\text{nm} \pm 11 \text{ nm}$; 362 spores analysed with a residual fitting error of 6.5%), and presumably CwlJ
213 (Setlow *et al.* 2017; Ghosh *et al.* 2018). In contrast, no fluorescence was observed during
214 sporulation or in mature spores for the BMQ_2391-GFP protein, perhaps due to aberrant protein

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3 215 folding or problems associated with the GFP fusion protein translocating the inner membrane (data
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5 216 not shown). A strain designed to express an N-terminal GFP fusion protein also failed to show any
6
7 217 detectable fluorescence during sporulation or in mature spores.
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219 **Germination of BMQ_2391 and BMQ_3234 null mutant spores**

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

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

247

248 **Interactions between CLEs**

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

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260 **Heterologous expression of BMQ_2391 and BMQ_3234**

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

268

269 **Discussion**

270 Initiation of cortex hydrolysis in current models of *Bacillus* spore germination requires the activity
271 of either of the semi redundant CLEs SleB or CwlJ. Efficient degradation of the cortex is
272 subsequently facilitated by enzymes such as SleL, whose function appears to be associated with
273 further hydrolysis of large peptidoglycan fragments generated by SleB and or CwlJ (Chen *et al.*
274 2000; Lambert & Popham 2008; Ustok *et al.* 2015). While this sequence of events probably occurs
275 in wild type spores of all species of *Bacillus*, mutagenesis analyses conducted with *B. megaterium*

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3 276 *sleB cwlJ* spores have revealed that this species can circumvent the requirement for either of SleB
4 277 or CwlJ . Previous work revealed that *B. megaterium sleB cwlJ* spores could degrade the cortex
5 278 with an efficiency comparable to wild type spores when complemented with a plasmid-borne copy
6 279 of *sleB^N*, which encodes the non catalytic domain of SleB, plus the adjacent *ypeB* gene (Christie
7 280 *et al.* 2010). Indeed, complementation with *ypeB* alone restored efficient cortex hydrolysis to *sleB*
8 281 *cwlJ* spores (Ustok *et al.* 2014). The precise function of YpeB has not been determined, although
9 282 it is not a peptidoglycan lysin, and instead appears to have a role in localising – and perhaps
10 283 regulating the activity - of SleB in the spore (Boland *et al.* 2000; Bernhards & Popham 2014; Ustok
11 284 *et al.* 2015).

12 285 Another study in *B. megaterium* extended these findings and attempted to dissect the
13 286 molecular mechanisms that support efficient cortex hydrolysis in *sleB cwlJ* spores (Ustok *et al.*
14 287 2014). In this case, a triple mutant (*sleB cwlJ sleL*) strain complemented with *sleB^N* plus *ypeB*
15 288 showed a severe germination defect. Based on this result, the study concluded that *ypeB* and *sleL*
16 289 are both essential for the initiation of cortex hydrolysis in *B. megaterium sleB cwlJ* spores. The
17 290 requirement for *sleL* in the *sleB cwlJ* background was unexpected since, as noted above, SleL is
18 291 regarded as a cortical fragment lytic enzyme. Similarly, mucopeptide analyses of germination
19 292 exudates from *sleB cwlJ* spores complemented with *ypeB* clearly indicate the presence of lytic
20 293 transglycosylase activity during germination, whereas SleL exhibits N-acetylglucosaminidase
21 294 activity (Lambert & Popham 2008; Ustok *et al.* 2014).

22 295 With this context in mind, the main purpose of the current study was to identify cortex lytic
23 296 enzymes that are functional in *B. megaterium sleB cwlJ* spores complemented with plasmid borne
24 297 *ypeB*. Progress in this regard was achieved via bioinformatic analyses, which identified five
25 298 candidate CLE loci. Subsequent transcriptional analyses, comprising RT-PCR and β -galactoside
26 299 reporter assays, were used to narrow candidates for further characterisation to proteins encoded at
27 300 the BMQ_2391 and BMQ_3234 loci (moderate transcription, relative to *sleB* and *cwlJ*, was
28 301 associated with BMQ_3195, although this protein was not characterised further).

29 302 Mutagenesis analyses in this work were compromised to a certain extent in that we could
30 303 not isolate null mutant strains that had undergone allelic exchange with truncated and disrupted
31 304 versions of *BMQ_2391* or *BMQ_3234* in the *sleB cwlJ* background. However, strains in which
32 305 *BMQ_2391* or *BMQ_3234* were disrupted by integrative plasmids, effectively separating the
33 306 promoter and first 300 nucleotides of the respective genes from the remainder of the coding

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3 307 sequences, were isolated, albeit at the expense of the GerU-encoding pBM600 plasmid. Loss of
4 308 the GerU germinant receptor can be circumvented by germinating *gerU* spores in beef extract,
5 309 components of which trigger efficient germination responses via alternative germinant receptors
6 310 in *B. megaterium* (Gupta *et al.* 2013). In both cases, germination of triple mutant spores – *sleB*
7 311 *cwlJ* *BMQ_2391* and *sleB cwlJ* *BMQ_3234* – complemented with plasmid borne *sleB^N* and *ypeB*,
8 312 was comparable in terms of absorbance loss to non *sleB^N ypeB* complemented triple mutant spores.
9 313 The observed reduction in A600 of approximately 15% is indicative of spores that have released
10 314 CaDPA and various ions from the spore core but which have failed to depolymerise cortical
11 315 peptidoglycan. Viability of the *sleB^N ypeB* complemented triple mutant spores was similarly
12 316 comparable to non-complemented spores, with colony forming ability on LB medium being
13 317 reduced by more than five logs compared to isogenic wild type spores, indicating again that the
14 318 cortex has not been degraded. In contrast, complementation in trans with *BMQ_2391* or
15 319 *BMQ_3234* in *sleB^N ypeB* complemented triple mutant spores restored viability and absorbance
16 320 loss to approximately 40% of wild type levels.

17 321 Outputs from the present study support the hypothesis that *BMQ_2391* and *BMQ_3234*
18 322 encode novel CLEs that are not only active during germination of *B. megaterium* spores, but which
19 323 also confer further redundancy within the cortical depolymerisation system of this species (and
20 324 perhaps this species alone since *sleB^N ypeB* in trans failed to restore cortex hydrolysis to *B. subtilis*
21 325 *sleB cwlJ* spores (Li *et al.* 2013)). In some regards the requirement for three separate enzymes -
22 326 SleL, *BMQ_2391* and *BMQ_3234* – in tandem with YpeB, for efficient spore germination in *B.*
23 327 *megaterium sleB cwlJ* spores, is difficult to reconcile. One possibility is that the combined activity
24 328 of each enzyme – which may be infrequent cutters – is required for cortex depolymerisation to a
25 329 degree that is commensurate with germination. It's possible also that at least some of these proteins
26 330 physically interact to ensure correct localisation and or function in the spore. Ellipsoid localisation
27 331 microscopy analysis places SleL and *BMQ_3234* within the same inner coat location within the
28 332 spore, which would facilitate physical interactions. However, where examined - bacterial two-
29 333 hybrid assays in this work and pull-down assays conducted previously (Li *et al.* 2013; Ustok *et al.*
30 334 2014) – have failed to provide any evidence of interactions between CLEs. Further insight to the
31 335 nature of the inter-dependency between CLEs, and how YpeB may contribute to this, are
32 336 objectives for continuing work in this area. Finally, targeted inactivation of CLEs may provide a
33 337 novel strategy for spore decontamination in a number of sectors. A potential implication arising

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3 338 from the present study is that enzymes in addition to the major CLEs may have to be inhibited, at
4
5 339 least in some species, for efficient spore inactivation.
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7 340

8 341 **Acknowledgements**
9

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11
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13
14 344 Sackler Foundation. Contributions to this work by Fatma Isik Ustok, Srishti Gupta, Ke Xu Zhou
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16 345 and Akshay Deshmukh are acknowledged.
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For Peer Review

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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 μ m.

Figure 3 Germination of *B. megaterium* spores in 5% (wt/vol) beef extract. Spores of the various strains were heat shocked (80°C for 30 min) and then cooled before incubating in beef extract, and absorbance (A_{600}) measurements recorded as described in the Materials and Methods. Key: GC103 ($\Delta sleB \Delta cwlJ$), crosses; GC106 ($\Delta sleB \Delta cwlJ$ pHT315-*sleB^N* *ypeB*), diamonds; BR111 ($\Delta sleB \Delta cwlJ \Delta BMQ_2391$ pHT-*sleB^N* *ypeB*) filled squares; BR113 ($\Delta sleB \Delta cwlJ \Delta BMQ_2391$ pHT-*sleB^N* *ypeB* *BMQ_2391*), open squares; BR112 ($\Delta sleB \Delta cwlJ \Delta BMQ_3234$ pHT-*sleB^N* *ypeB*), filled circles; BR114 ($\Delta sleB \Delta cwlJ \Delta BMQ_3234$ 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*.

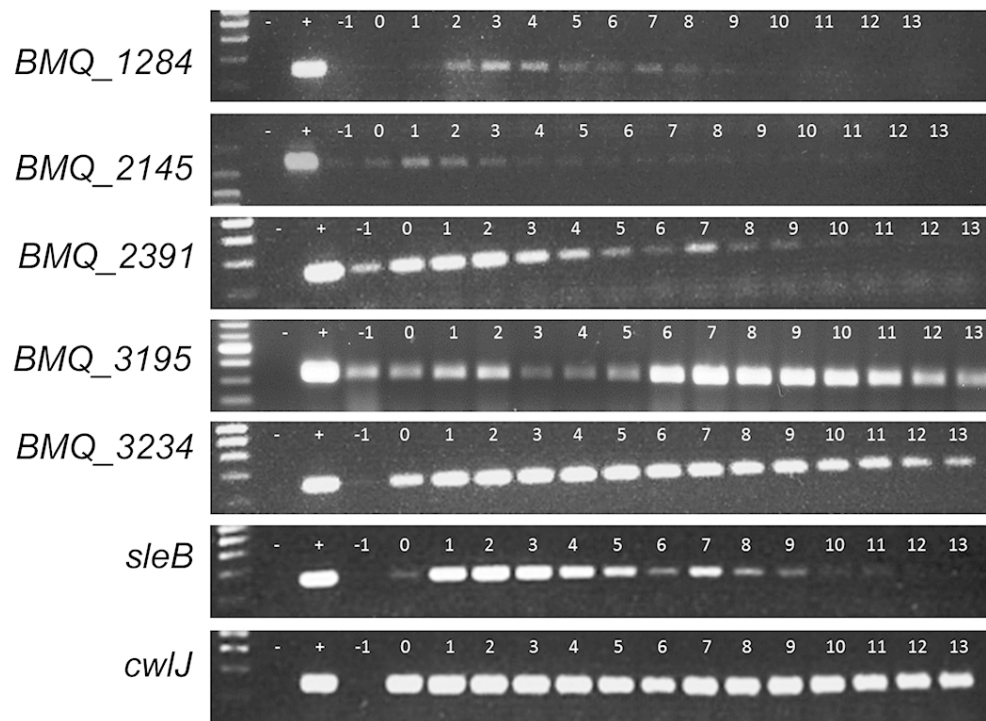


Figure 1 RT-PCR analysis of *sleB*, *cwIJ* and potential CLE gene transcription during sporulation of *B. megaterium* QM B1551. RT-PCR was conducted using gene-specific primers designed to amplify ~300-bp fragments of the respective genes from RNA isolated from sporulating cultures, as described in Materials 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).

90x67mm (300 x 300 DPI)

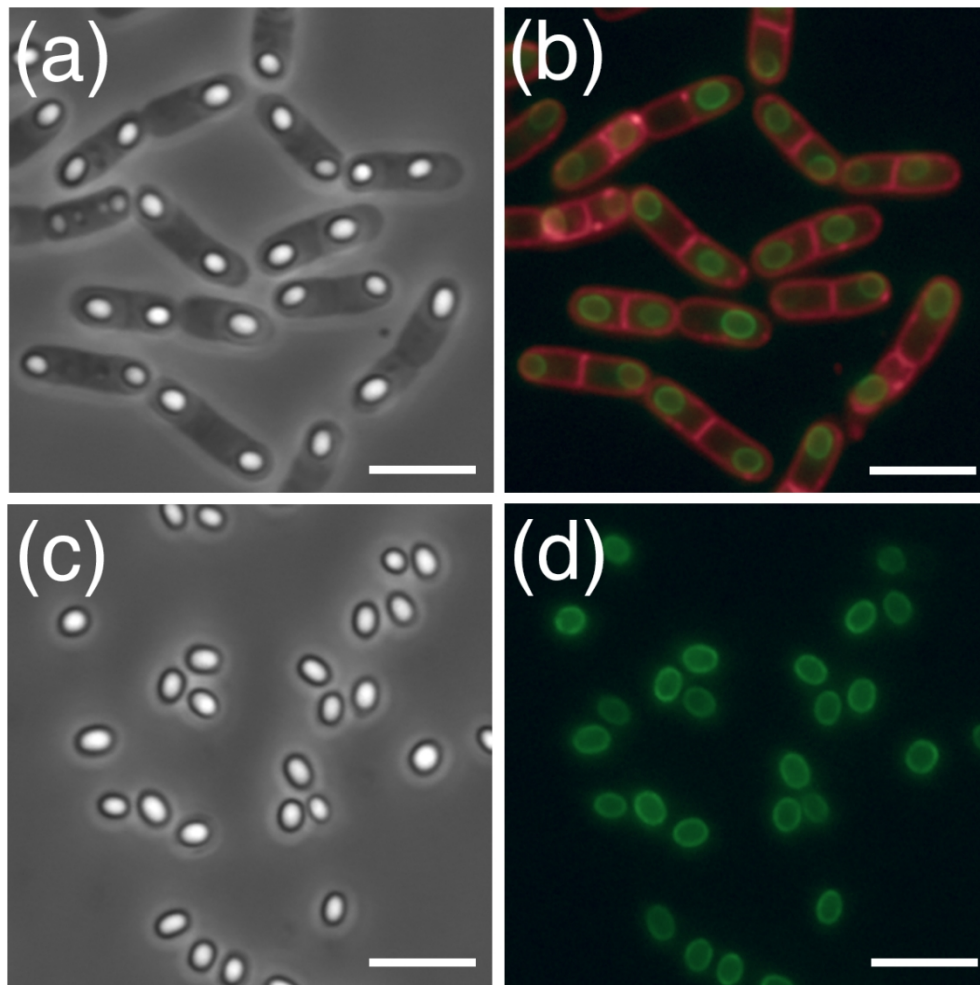


Figure 2 Phase contrast and fluorescence microscopy of *B. megaterium* *BMQ_3234-gfp* sporulating cells (a and b) and mature spores (c and d). Green fluorescence is associated with the *BMQ_3234-GFP* fusion protein and red fluorescence with the membrane-staining FM4-64 dye. Bar represents 5 μm.

103x102mm (300 x 300 DPI)

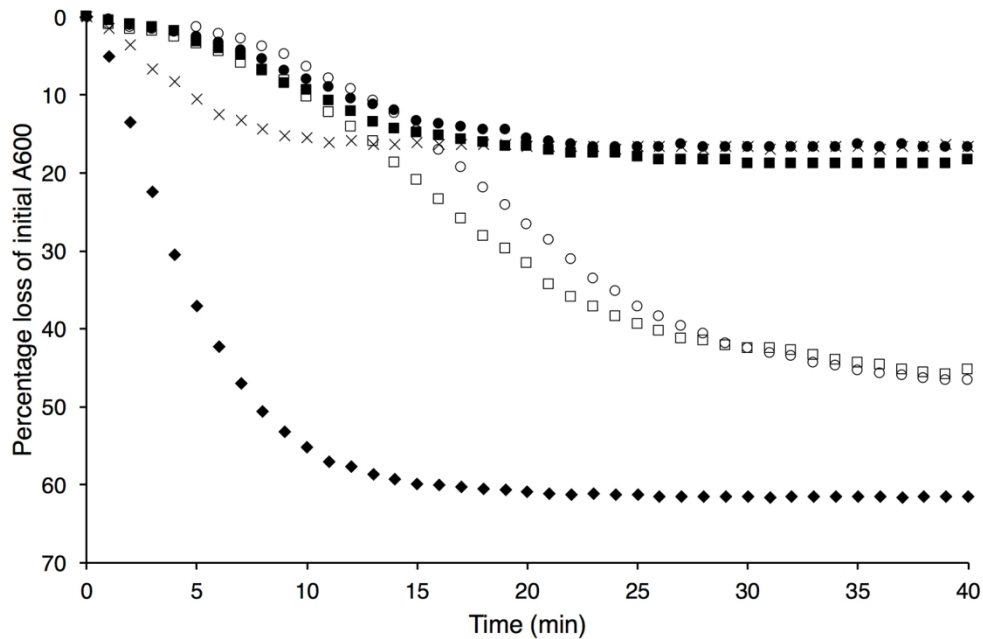


Figure 3 Germination of *B. megaterium* spores in 5% (wt/vol) beef extract. Spores of the various strains were heat shocked (80°C for 30 min) and then cooled before incubating in beef extract, and absorbance (A600) measurements recorded as described in the Materials and Methods. Key: GC103 (Δ sleB Δ 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.

150x97mm (300 x 300 DPI)

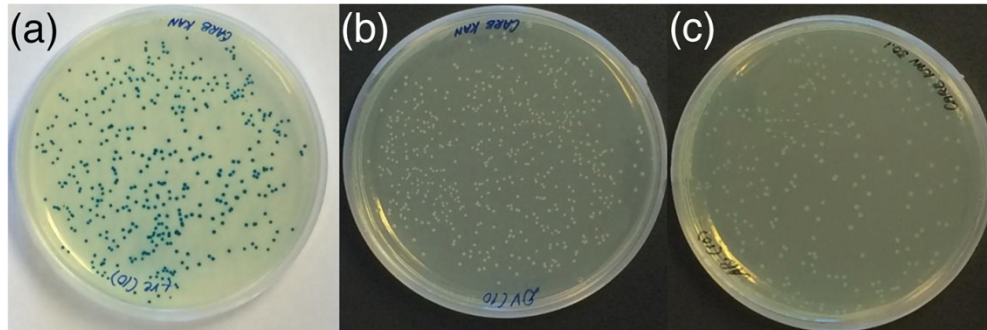


Figure 4 Sample output from bacterial adenylate cyclase two-hybrid analysis for interactions between *B. megaterium* CLEs. Positive interactions, denoted by blue *E. coli* colonies, were observed only for the manufacturer's positive control (a). White colonies – indicative of proteins that do not interact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

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	$\Delta sleB \Delta cwlJ$ Km ^r Sp ^r	0.001	—	(Christie <i>et al.</i> , 2010)
GC106	$\Delta sleB \Delta cwlJ$ pHT- <i>sleB</i> ^N <i>ypeB</i> Km ^r Sp ^r MLS ^r	80	—	(Christie <i>et al.</i> , 2010)
GC123	$\Delta sleB \Delta cwlJ$ pHT- <i>ypeB</i> Km ^r Sp ^r MLS ^r	50	—	(Ustok <i>et al.</i> , 2014)
BR111	$\Delta sleB \Delta cwlJ \Delta BMQ_{2391}$ pHT- <i>sleB</i> ^N <i>ypeB</i> Km ^r Sp ^r Tc ^r MLS ^r	<0.001	—	This study
BR112	$\Delta sleB \Delta cwlJ \Delta BMQ_{3234}$ pHT- <i>sleB</i> ^N <i>ypeB</i> Km ^r Sp ^r Tc ^r MLS ^r	<0.001	—	This study
BR113	$\Delta sleB \Delta cwlJ \Delta BMQ_{2391}$ pHT- <i>sleB</i> ^N <i>ypeB</i> <i>BMQ_{2391}</i> Km ^r Sp ^r Tc ^r MLS ^r	40	—	This study
BR114	$\Delta sleB \Delta cwlJ \Delta BMQ_{3234}$ pHT- <i>sleB</i> ^N <i>ypeB</i> <i>BMQ_{3234}</i> Km ^r Sp ^r Tc ^r MLS ^r	35	—	This study
BR115	$\Delta sleB \Delta cwlJ \Delta BMQ_{2391}$ pHT- <i>BMQ_{2391}</i> Km ^r Sp ^r Tc ^r MLS ^r	0.001	—	This study
BR116	$\Delta sleB \Delta cwlJ \Delta BMQ_{3234}$ pHT- <i>BMQ_{3234}</i> Km ^r Sp ^r Tc ^r MLS ^r	0.002	—	This study
lacZ fusion strains				
BR101	$\Delta BMQ_{1284}::pNFd13$ Km ^r	—	170	This study
BR102	$\Delta BMQ_{2145}::pNFd13$ Km ^r	—	210	This study
BR103	$\Delta BMQ_{2391}::pNFd13$ Km ^r	—	1115	This study

BR104	$\Delta BMQ_{3195}::pNFd13$ Km ^r	—	490	This study
BR105	$\Delta BMQ_{3234}::pNFd13$ Km ^r	—	1130	This study
BR106	$\Delta BMQ_{sleB}::pNFd13$ Km ^r	—	1950	This study
BR107	$\Delta BMQ_{cwlJ}::pNFd13$ Km ^r	—	1755	This study
<i>gfp</i> fusion strains				
BR108	<i>BMQ_{2391-gfp}</i> Cm ^r	—	—	This study
BR109	<i>BMQ_{3234-gfp}</i> Cm ^r	—	—	This study
BR110	<i>gfp-BMQ_{2391}</i> 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.

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3 Response to Reviewers' comments
4

5 Novel cortex lytic enzymes in *Bacillus megaterium* QM B1551 spores
6

7 Manuscript id: FEMSLE-19-03-0180
8

9 Authors: Al-Riyami, Bahja; Ghosh, Abhinaba; Rees, Eric; Christie, Graham
10

11 The authors appreciate the time and effort taken by the reviewers in considering our manuscript and
12 thank them for their useful suggestions, critiques and clarifications.
13
14

15
16 Reviewer: 1
17

18 This manuscript provides strong evidence on the identity of additional enzymes besides the two
19 known redundant cortex-lytic enzymes CwlJ and SleB can give sufficient cortex peptidoglycan
20 hydrolysis to allow completion of *B. megaterium* spore germination. There are, however, a number
21 of relatively minor concerns that need to be addressed as follows.
22

23 1) There are a number of examples of unusual word usage in the manuscript. Three early in the
24 manuscript are "initiate" on l 17, "consistent" on l 24 and "encompass" on l 31
25

26 Response

27 The abstract has been modified i.e. 'initiate' has been deleted, whereas the sentence with 'consistent'
28 now reads 'The findings reinforce the idea that there is a degree of variation in mechanisms of cortex
29 hydrolysis across the *Bacillales*, raising potential implications for environmental decontamination
30 strategies based upon targeted inactivation of components of the spore germination apparatus.' Line
31 31 has been changed from 'encompass' to 'include'.
32
33

34
35 2) The abstract never mentions YpeB, yet this alone is sufficient to allow cwlJ sleB spore to
36 germinate.
37

38 Response

39 The abstract has been modified to include the sentence: 'Previous work has demonstrated that *B.*
40 *megaterium* spores may differ to other species in this regard, since *sleB cwlJ* null mutant spores
41 complemented with the gene in *trans* for the non-peptidoglycan lysin YpeB can efficiently degrade
42 the cortex.'
43

44 3) The issue of the possible implications of the additional CLEs shown in this manuscript to
45 environmental decontamination is never mentioned again.
46

47 Response

48 A short section has been added to the end of the manuscript i.e. "Finally, targeted inactivation of
49 CLEs may provide a novel strategy for spore decontamination in a number of sectors. A potential
50 implication arising from the present study is that enzymes in addition to the major CLEs may have to
51 be inhibited, at least in some species, for efficient spore inactivation.'
52
53

54
55 4) l 39/40 - A little misleading here, in that all Clostridial spores have CLEs, just many don't have
56 CwlJ and SleB, but rather SleC.
57

58 Response
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60

1
2
3 Correct, that was an unintended consequence from a previous edit. The sentence now reads 'In spores
4 of all species examined the cortex is degraded by specialised peptidoglycan lysins, which are referred
5 to as cortex lytic enzymes (CLEs).'

6
7 5) l 56 - what is the copy number, and was the truncated SleB needed, and if not, what is the YpeB
8 level needed to get cwIJ sleB spores to germinate?
9

10 Response

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

16
17 6) How was YpeB complemented and what was the level in complemented spores?
18

19 Response

20 YpeB was complemented using a pHT315-derived plasmid. A reference to this work is now cited in
21 line 61.
22

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

24 Response

25 This sentence has been changed and the appropriate reference added to the manuscript:
26 'Complementation-based analyses of CLE mutant strains were performed using pHT315-derived
27 plasmids, which have a copy number of approximately 15 per cell (Arantes & Lereclus 1991).
28
29

30
31 8) Are you sure you have all upstream regulatory sequences, and if so, why?
32

33 Response

34 Promoter sequences with appropriate sigma factor recognition sequences were identified by sequence
35 analysis so we are confident that the regulatory sequences were captured. Successful
36 complementation analyses indicate also that the cloned ORFs plus upstream sequences were
37 expressed.
38
39

40 9) l 207 - is the fusion protein even expressed- as determined by a Western?
41

42 Response

43 We didn't check this but given that the upstream regulatory sequence was identical to that used in
44 complementation analyses it seems highly likely that the fusion protein was expressed.
45
46

47 10) Fig. 2, legend - state explicitly that the green fluorescence is due to the fusion protein, as the
48 parental strain shows no fluorescence.
49

50 Response

51 The appropriate sentence in the legend has been modified to read: 'Green fluorescence is associated
52 with the BMQ_3234-GFP fusion protein and red fluorescence with the membrane-staining FM4-64
53 dye.'
54
55

56 11) In Fig. 3, give the relevant genotype in the legend so readers don't have to go back to the strain
57 Table.
58

59 Response

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

1
2
3
4 12 Table 1 - were backgrounds subtracted from values for RFU in Table 1?
5

6 Response

7 Yes. The footnote now reads: 'β-galactosidase values (relative fluorescence units [RFU]) are the
8 average of triplicate measurements, with background values subtracted, conducted with two different
9 spore preparations.'
10

11 Reviewer: 2

12 Comments to the Author

13
14 This paper by Al-Riyami et al describes the identification of two apparent spore cortex lytic enzymes
15 that can play minor roles in *B. megaterium* spore germination. This data can help to explain
16 previously observed differences between this species and some other Bacilli, though, as the authors
17 note, it leaves unresolved, and even possibly complicates the question of how YpeB (and possibly the
18 N-terminal domain of SleB), which does not have lytic activity, facilitates the activity of other
19 enzymes that are not even expected to reside in the same location in the spore.
20

21 The methods are properly applied, the data are clearly presented, and the conclusions are mostly
22 justified. My concerns are with one major point of the complementation studies and with under-
23 presentation of bioinformatic analyses of the loci.
24

25
26 1. The inclusion of additional information regarding the new loci under study would help the
27 reader (and authors) to interpret the data.

28 a. Table 2: Do any of these proteins have predicted signal sequences? (For comparison, SleB
29 does and CwlJ does not.) This could also help with analysis of the GFP-fusion study (see below)
30

31 Response

32 The four SleB homologues have predicted signal sequences whereas the CwlJ homologue doesn't.
33 The manuscript has been updated to include the following sentences: 'Sequence analysis revealed
34 possible sigma G (σ^G) consensus sequences and predicted signal peptide sequences for all four SleB
35 homologues, which is consistent with expression in the forespore during sporulation. In contrast, the
36 BMQ_3234 ORF is preceded by a potential sigma E (σ^E) recognition sequence and lacks a
37 recognisable signal peptide, which is consistent with expression in the mother cell during
38 sporulation.' Table 2 has also been updated to include signal sequence information.
39

40 b. Are any of these loci in predicted operons? If so, it greatly complicates the complementation
41 studies. This might also inform the surprising inability to create deletion mutations in these genes and
42 the odd, perhaps obligatory, loss of GerU in the mutant strains.
43

44 Response

45 Neither BMQ_2391 nor BMQ_3234 are predicted to reside in operons. We're not sure why it proved
46 difficult to create deletion mutants in these genes, although we've experienced similar problems with
47 several other spore associated genes. Loss of the large plasmid that encodes GerU is also very
48 common during mutant construction. Ultimately, an improved mutagenesis method for *B. megaterium*
49 is required.
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52 2. Lines 206-210: The fact that a GFP fusion to BMQ_3234 works and a fusion to BMQ_2391
53 does not might be related to the localization of the protein. If BMQ_3234 is expressed in the mother
54 cell and localizes to the spore coats, as for CwlJ, the fusion protein would not have to cross a
55 membrane, similarly to CwlJ, for which a GFP fusion is functional. If BMQ_2391 has to cross a
56 membrane for incorporation into the mature spore, then GFP will not work, as for SleB. Do these
57 proteins have predicted signal sequences?
58

59 Response

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

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13 3. It is especially hard to explain how complementation with YpeB works along with
14 BMQ_3234, which is apparently in the spore coat, while YpeB is expected to be associated with the
15 inner spore membrane. This raises the question of whether YpeB (and SleBN) are actually needed for
16 this complementation. The complementation is done with a "low copy" plasmid, but what is the
17 actual copy number, and more importantly, what is the expression level of BMQ-2391 and
18 BMQ_3234 from this plasmid? It is possible that simply several-fold overexpression of these gene
19 products is sufficient to provide the cortex lysis needed to compete the observed slow germination.
20 The presence of SleBN and YpeB on the complementation plasmids might not be required at all! To
21 properly address this, complementation plasmids carrying the new novel genes alone should be tested.
22

23 Response

24 Two new strains were constructed to test this possibility (BR115 and BR116, added to Table 1). The
25 viability of these spores was not appreciably different to $\Delta sleB \Delta cwlJ$ spores indicating that YpeB is
26 required for efficient cortex hydrolysis in the $\Delta sleB \Delta cwlJ$ background as opposed to over-expression
27 of CLEs (the plasmid copy number is approximately 15 per cell). How YpeB influences CLEs that are
28 synthesised in the mother cell and deposited in the spore coat is a mystery, although the Moir lab
29 provided evidence via antibody labelling that YpeB has a dual location (coat and inner membrane;
30 equally, our own unpublished super resolution analyses indicate inner membrane only). A sentence
31 has been added to the manuscript: 'In contrast, the viability of triple mutant spores complemented
32 with either BMQ_2391 or BMQ_3234 was comparable to spores of the respective non-complemented
33 strains, indicating that YpeB is essential for cortical depolymerisation in the *sleB cwlJ* background.'
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3 1 **Novel cortex lytic enzymes in *Bacillus megaterium* QM B1551 spores**
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8 3 Bahja Al Riyami, Abhinaba Ghosh, Eric J. Rees, and Graham Christie*

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26 11 One sentence summary: Proteins encoded at BMQ_2391 and BMQ_3234 encode novel cortex
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28 12 lytic enzymes that permit cortex hydrolysis in *Bacillus megaterium* spores deficient in the major
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31 13 peptidoglycan lysins SleB and CwlJ.
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3 **15 Abstract**
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6 16 Present models for spore germination in *Bacillus* species include a requirement for either the SleB
7
8 17 or CwlJ cortex lytic enzymes to ~~initiate and~~ efficiently depolymerise the spore cortex. Previous
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10 18 work has demonstrated that *B. megaterium* spores may differ to other species in this regard, since
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12 19 *sleB cwlJ* null mutant spores complemented with ~~defined the gene in trans for the~~ non-
13
14 20 peptidoglycan lysin ~~genes in trans~~ *YpeB* can efficiently degrade the cortex. Here we identify two
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16 21 novel cortex lytic enzymes, encoded at the BMQ_2391 and BMQ_3234 loci, that are essential for
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18 22 cortex hydrolysis in the absence of SleB and CwlJ. Ellipsoid localisation microscopy places the
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20 23 BMQ_3234 protein within the inner-spore coat, a region of the spore that is populated by other
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22 24 cortex lytic enzymes. The findings reinforce the idea that there is a degree of variation in
23
24 25 mechanisms of cortex hydrolysis across the *Bacillales* ~~are not entirely consistent and raise, raising~~
25
26 26 potential implications for environmental decontamination strategies based upon targeted
27
28 27 inactivation of components of the spore germination apparatus.

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

31 Bacteria of the *Bacillales* and *Clostridiales* form metabolically dormant endospores (spores) in
32 response to nutrient limitation. The cellular properties of spores, which ~~encompass~~include several
33 unique morphological and physiological features, means that they are equipped to persist in the
34 environment, in a dormant state, until conditions are conducive to support vegetative growth. The
35 latter is indicated by the presence of nutrient molecules, typically amino acids, monosaccharides
36 and nucleosides, which interact with germinant receptor molecules to trigger spore germination
37 (Setlow *et al.* 2017). A major event in spore germination concerns depolymerisation of the spore
38 cortex, a thick layer of structurally unique peptidoglycan that envelops the spore protoplast and
39 which is a major contributory factor to the maintenance of spore dormancy (Imae & Strominger
40 1976; Popham & Bernhards 2015). In *Bacillus* spores of all species, and some *Clostridia*, examined
41 the cortex is degraded by specialised peptidoglycan lysins, which are referred to as cortex lytic
42 enzymes (CLEs). It is well established that two main CLEs, SleB and CwlJ, can initiate hydrolysis
43 of the cortex during the germination process (Moriyama *et al.* 1996; Ishikawa *et al.* 1998). SleB
44 and CwlJ are semi-redundant enzymes, in the sense that deletion of either gene permits cortex
45 hydrolysis to an extent where at least a proportion of the spores within a population will retain
46 viability on rich culture medium. Deletion of both genes, however, results in spores that are
47 severely compromised in their capacity to depolymerise the cortex resulting in arrested germination
48 and severely reduced colony forming ability (Ishikawa *et al.* 1998; Heffron *et al.* 2009; Setlow *et*
49 *al.* 2009).

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

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4 61 background (Christie *et al.* 2010). Structural analysis of cortical fragments produced during
5 62 germination of these spores, and in spores where only *ypeB* was complemented, which also
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7 63 germinate efficiently, ~~revealed a relative abundance of anhydromuropeptides in the germination~~
8
9 64 ~~milieu.~~ (Ustok *et al.* 2014), ~~revealed a relative abundance of anhydromuropeptides in the~~
10 65 ~~germination milieu.~~ These are the products of a class of peptidoglycan lysin referred to as a lytic
11
12 66 transglycosylase. Since SleB is a well characterised lytic transglycosylase (Boland *et al.* 2000;
13
14 67 Jing *et al.* 2012; Li *et al.* 2012), the obvious conclusion here is that an orthologue of SleB is present
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16 68 in *B. megaterium* spores, which – like SleB - has an undefined requirement for YpeB, and which
17
18 69 can initiate cortex depolymerisation in the absence of SleB and CwlJ.

19 70 Accordingly, the objective of the current study was to identify and characterise novel CLEs
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21 71 that are functional during the germination of *B. megaterium* QM B1551 spores, and in particular,
22
23 72 those that permit efficient cortex hydrolysis in the absence of SleB and CwlJ.

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25 74 **Materials and Methods**

26 75 **Bacterial strains and spore preparation**

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28 76 *B. megaterium* strains employed in this study, which were all isogenic with the QM B1551 strain,
29
30 77 were cultured routinely at 30°C on LB medium supplemented where appropriate with antibiotics
31
32 78 (Table 1). Spores were prepared by nutrient exhaustion in supplemented nutrient broth and
33
34 79 subsequently purified by repeated rounds of centrifugation and resuspension of spore pellets in
35
36 80 deionised ice-cold water (Christie *et al.* 2010). Purified suspensions comprising >99% phase bright
37
38 81 spores were stored in deionised water, at an A600 of approximately 50, on ice. *Escherichia coli*
39
40 82 Top 10 cells (Thermo Fisher, UK) were used for cloning procedures, plasmid propagation and for
41
42 83 heterologous protein expression experiments.

43 84

44 85 **Spore germination and viability assays**

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46 86 Spore germination in liquid medium was assessed in microtitre plates by adding 10 µl of heat-
47
48 87 shocked (80°C, 30 min) spores to 190 µl of 5% (wt/vol) beef extract (Oxoid, Ltd., Basingstoke,
49
50 88 United Kingdom), preheated to 37°C. The A600 of spore suspensions was 0.4. Plates were sealed
51
52 89 with adhesive film to minimize evaporative losses, and then incubated in a Perkin-Elmer
53
54 90 EnVision-Xcite multilabel plate reader fitted with a 600 nm photometric filter. Plates were agitated
55
56 91 orbitally for 10 seconds prior to absorbance measurements, which were recorded every minute for

92 90 minutes. Experiments were conducted in triplicate, with at least two or more independent spore
93 preparations. Spore viability was determined by pipetting 10 μ l aliquots of serially diluted
94 suspensions of heat-shocked spores (A600 of 1 [10^8 spores/ml]) onto LB agar plates. Plates were
95 incubated for 16 to 24 h prior to colony enumeration.

97 **Molecular biology procedures**

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

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

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

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4 123 ~~number pHT315-derived plasmids.~~ pHT315-derived plasmids, which have a copy number of
5 124 approximately 15 per cell (Arantes & Lereclus 1991). The existing pHT-*sleB^N* *ypeB* plasmid
6
7 125 (Christie *et al.* 2010) was modified to additionally incorporate ORFs and upstream regulatory
8
9 126 sequences for either BMQ_2391 or BMQ_3234 proteins. This was achieved using EcoRI restricted
10
11 127 pHT-*sleB^N* *ypeB* and BMQ_2391 or BMQ_3234 PCR amplicons designed to facilitate cloning via
12
13 128 the Klenow Assembly Method (Ghosh *et al.* 2018). Similar procedures were used to introduce
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15 129 BMQ_2391 or BMQ_3234 ORFs and regulatory sequences into pHT315. The *B. megaterium sleB*
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17 130 *cwlJ* BMQ_2391 and *B. megaterium sleB cwlJ* BMQ_3234 strains were subsequently transformed,
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19 131 using the appropriate ~~plasmid~~ plasmids, to erythromycin and lincomycin resistance.
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22 133 **Ellipsoid localisation microscopy**

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24 134 The quantitative fluorescence ELM technique was used to measure the location of GFP fusion
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26 135 proteins in mature spores (Manetsberger *et al.* 2015; Manton *et al.* 2018). Briefly, several
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28 136 independent fields of GFP labelled spores were imaged using an Olympus BX53 microscope fitted
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30 137 with a 100X 1.30 NA oil objective lens, with illumination from a mercury lamp, filters for GFP
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32 138 fluorescence, and a Retiga 2000R CCD camera. Automated image segmentation was used to
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34 139 identify single spores, and the image of each candidate was used to fit the parameters of a model
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36 140 that describes the image of a spheroidal fluorescent shell. For *B. megaterium*, an equation
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38 141 describing the image of a spherical fluorescent layer was fitted to the image data. A filter was
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40 142 applied to exclude fits from overlapping spores and fragments of debris. The average radius
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42 143 parameter fitted to the spores provides an estimate of the midpoint radial position of the GFP fusion
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44 144 with respect to the spore centre.
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47 146 **Protein interactions**

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49 147 A bacterial adenylate cyclase two-hybrid (BACTH) system (Euromedex, Souffelweyersheim,
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51 148 France) was used to characterise potential interactions *in vivo* between *B. megaterium* CLEs and
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53 149 related proteins (BMQ_2391, BMQ_3234, CwlJ, SleB, SleL and YpeB). The BACTH system is
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55 150 based upon two complementary adenylate cyclase fragments (T25 and T18) being brought together
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57 151 by interacting fusion partners to form functional enzyme, resulting in cAMP synthesis and
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59 152 activation of a *lacZ* reporter gene. Plasmids used for BACTH assays in this work were prepared
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153 by PCR amplifying ORFs encoding CLEs and YpeB from genomic DNA before assembling with

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3 154 linearised pUT18 and pKT25 plasmids by Klenow assembly. The resulting plasmids, designed for
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5 155 expression of C-terminal fusions between proteins of interest and T25 or T18 adenylate cyclase
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7 156 fragments, were isolated and verified by sequencing. Electrocompetent *E. coli* BTH101 were co-
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9 157 transformed using various combinations of pKT25 and pUT18 derived plasmids and selected on
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11 158 LB agar plates supplemented with 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-
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13 159 galactopyranoside), 1 mM IPTG, and 50 µg/ml carbenicillin and kanamycin, respectively. Cells
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15 160 were cultured for 24-72 h at 30°C and examined for the development of blue coloration, which is
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17 161 indicative of positive interactions between pairs of fusion proteins.
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For Peer Review

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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).

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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

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3 193 sporulation, whereas BMQ_3234 appears to be transcribed strongly throughout. The latter pattern
4
5 194 of transcription matches that of *cwlJ*. Further transcriptional analyses of candidate and established
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7 195 CLE promoters were achieved by placing the *lacZ* gene under control of putative promoter
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9 196 sequences for the various genes. The outputs from β -galactosidase assays conducted with
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11 197 supernatants from disrupted spores of the various *lacZ* reporter strains are broadly in line with
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13 198 results from the aforementioned RT-PCR assays i.e. where BMQ_1284 and BMQ_2145
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15 199 transcriptional fusions are associated with little β -galactosidase activity, while BMQ_2391 and
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17 200 BMQ_3234 show the highest levels of expression, albeit less than evident for *sleB* and *cwlJ*
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19 201 transcriptional fusions (Table 1).

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202 **Localisation of BMQ_2391 and BMQ_3234 GFP fusion proteins**

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23 204 Proteins encoded at the BMQ_2391 and BMQ_3234 loci were selected for further analysis since
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25 205 they appeared to be expressed at the highest levels of the candidate CLEs. The sub-cellular
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27 206 localisation of both proteins was investigated during sporulation and in mature spores by creating
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29 207 constructs designed to express C-terminal GFP fusion proteins. Fluorescence microscopy revealed
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31 208 that the BMQ_3234-GFP protein is expressed during sporulation in the mother cell compartment
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33 209 and is deposited to form a ring of fluorescence around the developing forespore (Figure 2). A
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35 210 strong fluorescence signal was retained in mature spores (Figure 2). The average radial location of
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37 211 the BMQ_3234-GFP protein, as inferred from ellipsoid localisation analyses, was 521 nm \pm 9 nm
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39 212 in mature spores (where \pm 9 nm is the standard deviation of radial locations found in repeated
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41 213 measurements and where 865 spores were analysed with a residual fitting error of 8.4%). This
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43 214 places the location of BMQ_3234 to the inner spore coat, within the same vicinity as SleL (525
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45 215 nm \pm 11 nm; 362 spores analysed with a residual fitting error of 6.5%), and presumably CwlJ
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47 216 (Setlow *et al.* 2017; Ghosh *et al.* 2018). In contrast, no fluorescence was observed during
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49 217 sporulation or in mature spores for the BMQ_2391-GFP protein, ~~presumably perhaps~~ due to
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51 218 aberrant protein folding or ~~mis-localisation of problems associated with~~ the GFP fusion protein
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53 219 ~~translocating the inner membrane~~ (data not shown). ~~Consequently, another A~~ strain was
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55 220 ~~constructed, in this case~~ designed to express an N-terminal GFP fusion protein. ~~Again, also failed~~
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57 221 ~~to show any detectable~~ fluorescence ~~signal was not detected throughout during~~ sporulation or in
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59 222 mature spores.
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224 Germination of BMQ_2391 and BMQ_3234 null mutant spores

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

253 Interactions between CLEs

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3 254 Results with null mutant spores reported in this work, and in previous studies (Bernhards &
4 255 Popham 2014; Ustok *et al.* 2014), indicate that direct physical interactions between CLEs and or
5 256 CLEs and the YpeB protein may be required to facilitate efficient cortex hydrolysis during
6 257 germination. The *E. coli* based BACTH system was employed in the current study to investigate
7 258 potential interactions between the two novel CLEs identified in this work – BMQ_2391 and
8 259 BMQ_3234 – with each other and with SleB, CwlJ, SleL and YpeB. Potential interactions
9 260 between (i) SleB with YpeB, SleL and CwlJ, (ii) CwlJ with YpeB and SleL, and (iii) SleL with
10 261 YpeB, were also examined. Blue coloration of co-transformant *E. coli* colonies was not observed
11 262 in any of the combinations tested, with the exception of the positive control, indicating that none
12 263 of the spore proteins of interest interact productively in this system (Figure 4).
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22 265 **Heterologous expression of BMQ_2391 and BMQ_3234**

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24 266 Several attempts were made to express recombinant versions of the BMQ_2391 and BMQ_3234
25 267 proteins with a view to characterising the hydrolytic bond specificity of each protein. Hosts for
26 268 heterologous expression included *E. coli*, *Lactococcus lactis* and *B. subtilis*, with several variant
27 269 proteins – including truncated forms, GFP, and maltose binding protein (solubility enhancer)
28 270 fusions – being examined over the course of this work. Unfortunately, levels of expression
29 271 commensurate with biochemical analyses were not achieved in any of the systems employed (data
30 272 not shown).
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38 274 **Discussion**

39 275 Initiation of cortex hydrolysis in current models of *Bacillus* spore germination requires the activity
40 276 of either of the semi redundant CLEs SleB or CwlJ. Efficient degradation of the cortex is
41 277 subsequently facilitated by enzymes such as SleL, whose function appears to be associated with
42 278 further hydrolysis of large peptidoglycan fragments generated by SleB and or CwlJ (Chen *et al.*
43 279 2000; Lambert & Popham 2008; Ustok *et al.* 2015). While this sequence of events probably occurs
44 280 in wild type spores of all species of *Bacillus*, mutagenesis analyses conducted with *B. megaterium*
45 281 *sleB cwlJ* spores have revealed that this species can circumvent the requirement for either of SleB
46 282 or CwlJ . Previous work revealed that *B. megaterium sleB cwlJ* spores could degrade the cortex
47 283 with an efficiency comparable to wild type spores when complemented with a plasmid-borne copy
48 284 of *sleB^N*, which encodes the non catalytic domain of SleB, plus the adjacent *ypeB* gene (Christie
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3 285 *et al.* 2010). Indeed, complementation with *ypeB* alone restored efficient cortex hydrolysis to *sleB*
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5 286 *cwlJ* spores (Ustok *et al.* 2014). The precise function of YpeB has not been determined, although
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7 287 it is not a peptidoglycan lysin, and instead appears to have a role in localising – and perhaps
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9 288 regulating the activity - of SleB in the spore (Boland *et al.* 2000; Bernhards & Popham 2014; Ustok
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11 289 *et al.* 2015).

12 290 Another study in *B. megaterium* extended these findings and attempted to dissect the
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14 291 molecular mechanisms that support efficient cortex hydrolysis in *sleB cwlJ* spores (Ustok *et al.*
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16 292 2014). In this case, a triple mutant (*sleB cwlJ sleL*) strain complemented with *sleB^N* plus *ypeB*
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18 293 showed a severe germination defect. Based on this result, the study concluded that *ypeB* and *sleL*
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20 294 are both essential for the initiation of cortex hydrolysis in *B. megaterium sleB cwlJ* spores. The
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22 295 requirement for *sleL* in the *sleB cwlJ* background was unexpected since, as noted above, SleL is
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24 296 regarded as a cortical fragment lytic enzyme. Similarly, mucopeptide analyses of germination
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26 297 exudates from *sleB cwlJ* spores complemented with *ypeB* clearly indicate the presence of lytic
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28 298 transglycosylase activity during germination, whereas SleL exhibits N-acetylglucosaminidase
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299 activity (Lambert & Popham 2008; Ustok *et al.* 2014).

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31 300 With this context in mind, the main purpose of the current study was to identify cortex lytic
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33 301 enzymes that are functional in *B. megaterium sleB cwlJ* spores complemented with plasmid borne
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35 302 *ypeB*. Progress in this regard was achieved via bioinformatic analyses, which identified five
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37 303 candidate CLE loci. Subsequent transcriptional analyses, comprising RT-PCR and β -galactoside
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39 304 reporter assays, were used to narrow candidates for further characterisation to proteins encoded at
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41 305 the BMQ_2391 and BMQ_3234 loci (moderate transcription, relative to *sleB* and *cwlJ*, was
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43 306 associated with BMQ_3195, although this protein was not characterised further).

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45 307 Mutagenesis analyses in this work were compromised to a certain extent in that we could
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47 308 not isolate null mutant strains that had undergone allelic exchange with truncated and disrupted
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49 309 versions of *BMQ_2391* or *BMQ_3234* in the *sleB cwlJ* background. However, strains in which
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51 310 *BMQ_2391* or *BMQ_3234* were disrupted by integrative plasmids, effectively separating the
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53 311 promoter and first 300 nucleotides of the respective genes from the remainder of the coding
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55 312 sequences, were isolated, albeit at the expense of the GerU-encoding pBM600 plasmid. Loss of
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57 313 the GerU germinant receptor can be circumvented by germinating *gerU* spores in beef extract,
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59 314 components of which trigger efficient germination responses via alternative germinant receptors
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315 in *B. megaterium* (Gupta *et al.* 2013). In both cases, germination of triple mutant spores – *sleB*

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3 316 *cwlJ* *BMQ_2391* and *sleB cwlJ* *BMQ_3234* – complemented with plasmid borne *sleB^N* and *ypeB*,
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5 317 was comparable in terms of absorbance loss to non *sleB^N ypeB* complemented triple mutant spores.
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7 318 The observed reduction in A600 of approximately 15% is indicative of spores that have released
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9 319 CaDPA and various ions from the spore core but which have failed to depolymerise cortical
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11 320 peptidoglycan. Viability of the *sleB^N ypeB* complemented triple mutant spores was similarly
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13 321 comparable to non-complemented spores, with colony forming ability on LB medium being
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15 322 reduced by more than five logs compared to isogenic wild type spores, indicating again that the
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17 323 cortex has not been degraded. In contrast, complementation in trans with *BMQ_2391* or
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19 324 *BMQ_3234* in *sleB^N ypeB* complemented triple mutant spores restored viability and absorbance
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21 325 loss to approximately 40% of wild type levels.

22 326 Outputs from the present study support the hypothesis that *BMQ_2391* and *BMQ_3234*
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24 327 encode novel CLEs that are not only active during germination of *B. megaterium* spores, but which
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26 328 also confer further redundancy within the cortical depolymerisation system of this species (and
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28 329 perhaps this species alone since *sleB^N ypeB* in trans failed to restore cortex hydrolysis to *B. subtilis*
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30 330 *sleB cwlJ* spores (Li *et al.* 2013)). In some regards the requirement for three separate enzymes -
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32 331 SleL, *BMQ_2391* and *BMQ_3234* – in tandem with YpeB, for efficient spore germination in *B.*
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34 332 *megaterium sleB cwlJ* spores, is difficult to reconcile. One possibility is that the combined activity
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36 333 of each enzyme – which may be infrequent cutters – is required for cortex depolymerisation to a
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38 334 degree that is commensurate with germination. It's possible also that at least some of these proteins
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40 335 physically interact to ensure correct localisation and or function in the spore. Ellipsoid localisation
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42 336 microscopy analysis places SleL and *BMQ_3234* within the same inner coat location within the
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44 337 spore, which would facilitate physical interactions. However, where examined - bacterial two-
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46 338 hybrid assays in this work and pull-down assays conducted previously (Li *et al.* 2013; Ustok *et al.*
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48 339 2014) – have failed to provide any evidence of interactions between CLEs. Accordingly,
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50 340 furtherFurther insight to the nature of the inter-dependency between CLEs, and how YpeB may
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52 341 contribute to this, are objectives for continuing work in this area. Finally, targeted inactivation of
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54 342 CLEs may provide a novel strategy for spore decontamination in a number of sectors. A potential
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56 343 implication arising from the present study is that enzymes in addition to the major CLEs may have
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58 344 to be inhibited, at least in some species, for efficient spore inactivation.
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For Peer Review

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5 352 **Conflicts of interest**
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7 353 None declared
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