Food Microbiology 84 (2019) 103259

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Biochemical and mutational analysis of spore cortex-lytic enzymes in the food spoiler *Bacillus licheniformis*

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ARTICLE INFO

Keywords:
Bacillus licheniformis
Spore viability
Spore germination
Cortex-lytic enzymes

ABSTRACT

Bacillus licheniformis is frequently associated with food spoilage due to its ability to form highly resistant endospores. The present study reveals that *B. licheniformis* spore peptidoglycan shares a similar structure to spores of other species of *Bacillus*. Two enzymatic activities associated with depolymerisation of the cortical peptidoglycan, which represents a crucial step in spore germination, were detected by muropeptide analysis. These include lytic transglycosylase and *N*-acetylglucosaminidase activity, with non-lytic epimerase activity also being detected. The role of various putative cortex-lytic enzymes that account for the aforementioned activity was investigated by mutational analysis. These analyses indicate that SleB is the major lysin involved in cortex depolymerisation in *B. licheniformis* spores, with CwlJ and SleL having lesser roles. Collectively, the results of this work indicate that *B. licheniformis* spores employ a similar approach for cortical depolymerisation during germination as spores of other *Bacillus* species.

1. Introduction

Bacillus licheniformis is an economically valuable bacterium due to its production of compounds such as enzymes, antibiotics, and surfactants that are used for various industrial purposes (Schallmey et al., 2004; Singh et al., 2016). It belongs to the *Bacillus subtilis* group and, as with other species belonging to this group, it is able to form endospores (spores) which are highly resistant to heat, chemicals, irradiation, and desiccation. *Bacillus* spores are usually formed under nutrient-limited conditions. Environmental cues that are indicative of conditions conducive to vegetative growth – typically involving an amino acid component - stimulate spore germination, whereby the bacteria exit the dormant state and resume vegetative growth (Setlow, 2003; Moir, 2006; Moir and Cooper, 2015). *B. licheniformis* spores are frequent contaminants of a wide range of foodstuffs. Correspondingly, if the foodstuff environment promotes germination then the resulting vegetative cells may subsequently cause food spoilage (Crielly et al., 1994; Thompson et al., 1998; Carlin, 2011; Andre et al., 2013). A modified tyndallisation process is utilized by the food industry to decrease the bacterial spore load in foods that minimises heat-associated loss of organoleptic properties of the product. The method employs a primary heat treatment at a moderate temperature which triggers spore germination, followed by a second moderate heat treatment that kills the germinated spores (Lovdal et al., 2011). Previous work has indicated that *B. licheniformis* spores germinate at a much slower rate than spores of other members of the *B. subtilis* group. This delayed germination property may explain why this species shows increased resistance to heat treatment procedures such as tyndallisation (Lovdal et al., 2013).

The key to spores' unique resistance properties lies in their complex and multi-layered structure. The coat layer provides much of the chemical and enzymatic resistance, beneath which is a thick layer of specialized peptidoglycan called the cortex. The latter contributes towards the reduced hydration status of the protoplast, or spore core, and is important for spore resistance to high temperature. A second layer of peptidoglycan, the germ cell wall, is present under the cortex, and is destined to develop into the cell wall of the vegetative bacterium after germination. Finally, the innermost layer comprises of a membrane that is characterized by low lipid mobility and which envelops the spore core to function as a permeability barrier against damaging chemicals (Setlow, 2006).

A crucial step in spore germination concerns degradation of the peptidoglycan cortex, which allows the spore core to rehydrate and expand. This process requires the activity of germination specific

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https://doi.org/10.1016/j.fm.2019.103259
Received 12 March 2019; Received in revised form 1 July 2019; Accepted 3 July 2019
Available online 04 July 2019

cortex-lytic enzymes (CLEs). The CLEs SleB and CwlJ play the most prominent role in cortex degradation among species belonging to the genus *Bacillus*. SleB consists of an *N*-terminal peptidoglycan-binding domain, a linker sequence and a C-terminal catalytic domain. The catalytic domain of SleB structurally resembles family-1 lytic transglycosylases, containing the conserved catalytic glutamate residue present in enzymes belonging to this family. However, SleB lacks motifs important in the interaction between transglycosylases and vegetative peptidoglycan substrates. Furthermore, SleB also differs from characterized transglycosylases in the arrangement of secondary-structure elements in the substrate-binding groove (Li et al., 2012). The *sleB* gene is transcribed, typically as part of an operon with *ypeB*, under control of the spore specific sigma factor σ^G, which is located in the forespore compartment. After synthesis, SleB is translocated across the inner membrane where it appears to be localized to the inner spore coat and perhaps the inner membrane (Moriyama et al., 1999; Chirakkal et al., 2002). SleB and YpeB are dependent on each other for localisation in the spore, although the precise function of YpeB has not been determined.

The precise catalytic specificity of CwlJ in the depolymerisation of cortical peptidoglycan has not been established but sequence homology with SleB, including the conserved single catalytic glutamate residue, indicates that it too is a lytic transglycosylase (Jing et al., 2012; Li et al., 2012). The *cwlJ* gene is transcribed in the mother cell, often as part of an operon with *gerQ*, and under the control of σ^E (Ishikawa et al., 1998; Moriyama et al., 1999). CwlJ appears to be localized to the inner spore coat, close to its cortical peptidoglycan substrate (Bagyan and Setlow, 2002). CaDPA, a 1:1 chelate of dipicolinic acid [DPA] with Ca^{2+}, is a chemical compound that comprises 5%–15% of the spore dry weight and contributes to spore resistance and dormancy (Setlow, 2006). CwlJ appears to be activated during germination by the efflux of CaDPA from the spore core but it can also be activated by high concentrations of exogenous CaDPA (Paidhungat et al., 2001; Setlow, 2003).

The *cwlJ2* gene (also named *ykvT*) encodes a protein (CwlJ2/YkvT) with strong homology to the SleB/CwlJ family of cortex lytic enzymes. The role of CwlJ2 in spore germination appears to differ among *Bacillus* species. *B. subtilis* spores lacking *cwlJ2*, for example, show comparable germinability to the corresponding wild type spores, while its absence in *B. anthracis* spores leads to significantly reduced germination and virulence (Chirakkal et al., 2002; Giebel et al., 2009).

The *sleL* (*yaaH*) gene was first described in *B. subtilis*, where it was shown to be transcribed under control of σ^E. As with CwlJ, SleL is located in the inner spore coat. SleL has *N*-acetylglucosaminidase activity but appears to act on cortical fragments as opposed to intact cortex. Structurally, SleL is comprised of two *N*-terminal LysM domains, which are probably involved in anchoring the protein to the substrate, and a C-terminal hydrolase domain that belongs to the family-18 glycosyl hydrolases (Lambert et al., 2012; Ustok et al., 2015). The crystal structures of *B. cereus* and *B. megaterium* SleL have been solved (Ustok et al., 2015), yielding some insight to differing rates of SleL activity observed in these two species. *B. anthracis sleL* null mutant spores do not show any significant germination deficiencies compared to wild type spores suggesting that the activity of other CLEs is sufficient to permit rapid spore germination and vegetative outgrowth (Lambert and Popham, 2008).

Despite its importance as a food spoilage organism, CLE activity during germination of *B. licheniformis* spores has not been examined in detail. The present study addresses this deficiency, examining the role of SleB, CwlJ, CwlJ2 and SleL in *B. licheniformis* spore germination, and extending our knowledge of the interplay and functional diversity of CLEs in spores of different *Bacillus* species.

Table 1
B. licheniformis strains and mutants used in this work.

Strain	Relevant genotype	Reference
ATCC14580/DSM13	Type strain	(Rey et al., 2004; Veith et al., 2004)
MW3	DSM13 Δ*hsdR1* Δ*hsdR2*	(Waschkau et al., 2008)
NVH-1331	Δ*sleB*	Kollerud (2014)
NVH-1333	Δ*cwlJ*	Kollerud (2014)
NVH-1341	Δ*sleB* Δ*cwlJ*	This study
NVH-1384	Δ*cwlJ* Δ*sleB* Δ*cwlJ2*	This study
NVH-1388	Δ*cwlJ2*	This study
NVH-1430	Δ*sleL*	This study
NVH-1432	Δ*sleB* Δ*cwlJ* Δ*cwlJ2* Δ*sleL*	This study
NVH-1372	NVH-1341 pHT315-*sleB*^{N}-*cwlJ*	This study
NVH-1374	NVH-1341 pHT315-*cwlJ*	This study
NVH-1375	NVH-1341 pHT315-*sleB*	This study
NVH-1379	NVH-1341 pHT315-*cwlJ*^{P}-*sleB*	This study
NVH-1469	NVH-1341 pHT315	This study
NVH-1470	NVH-1341 pHT315-*sleB ypeB*	This study

2. Materials and methods

2.1. Strains and growth conditions

Bacterial strains used in this work are listed in Table 1. *B. licheniformis* strains were grown in brain heart infusion media (BHI) (Difco), and *Escherichia coli* strains cultured in lysogeny broth (LB), supplemented with agar where required. Sporulation and purification of spores was performed as describe previously (Lovdal et al., 2012). This purification protocol gives a homogeneous suspension of phase bright spores without traces of vegetative cells (Supplementary Fig. S1). Purified spores were stored in deionised water at 4 °C prior to use in germination experiments.

2.2. Quantitative RT-PCR

Quantitative RT-PCR experiments were performed on mRNA isolated from *B. licheniformis* cultures harvested at three, five, eight and 21 h post-inoculation. At 21 h the degree of sporulation in the cultures was ~50% as judged by phase contrast microscopy. Sporulation for RNA extraction, cDNA synthesis and RT-qPCR analysis was performed as described before (Madslien et al., 2014). Each qPCR of the RNA samples was performed in triplicate, no template was added in negative controls, and *rpoB* was used as internal control. The qPCR analysis was performed on three independent biological replicates. Slopes of the standard curves and PCR efficiency (E) for each primer pair were estimated by amplifying serial dilutions of the cDNA template. For quantification of mRNA transcript levels, Ct (threshold cycle) values of the target genes and the internal control gene (*rpoB*) derived from the same sample in each real-time PCR reaction were first transformed using the term E-Ct. The expression levels of target genes were then normalized by dividing their transformed Ct-values by the corresponding values obtained for the internal control gene (Pfaffl, 2001; Duodu et al., 2010; Madslien et al., 2014). All primers used for RT-qPCR analyses are listed in Table 1S.

2.3. Construction of deletion mutants

The *B. licheniformis* strain MW3 was used as background for all mutants. The *sleB*, *cwlJ*, *cwlJ2* and *sleL* genes were deleted in-frame by replacing the reading frames with ATGTAA (5′–3′) using a markerless gene replacement method (Janes and Stibitz, 2006) with minor modifications. The Δ*sleB* Δ*cwlJ* double mutant, the Δ*sleB* Δ*cwlJ* Δ*cwlJ2* triple

mutant and the *ΔsleB ΔcwlJ ΔcwlJ2 ΔsleL* quadruple mutant strains were constructed using the same approach. To create the deletion mutants the regions upstream (primer A and B, Supplementary Table 1S) and downstream (primer C and D, Table Supplementary 1S) of the target genes were amplified. To allow assembly of the PCR fragments, primers B and C contained complementary overlapping sequences. An additional PCR step was then performed, using the upstream and downstream PCR fragments as template and the A and D primer pair (Supplementary Table 1S). All PCR reactions were conducted using an Eppendorf Mastercycler gradient and high fidelity AccuPrime Taq DNA Polymerase (ThermoFisher Scientific) according to the manufacturer's instructions. The final amplicons were cloned into the thermosensitive shuttle vector pMAD (Arnaud et al., 2004) containing an additional I-SceI site (a kind gift from Dr Annette Fagerlund, Nofima, Norway) as previously described (Lindback et al., 2012). The pMAD-I-*Sce*I plasmid constructs were introduced into *B. licheniformis* MW3 by electroporation (Mahillon et al., 1989). After verification of transformants, the plasmid pBKJ233, containing the gene for the I-*Sce*I enzyme, was introduced into the transformant strains by electroporation. The I-*Sce*I enzyme makes a double-stranded DNA break in the chromosomally integrated plasmid. Subsequent, homologous recombination mechanisms leads to excision of the integrated plasmid resulting in the desired genetic replacement. The gene deletions were verified by PCR amplification using primers A and D (Table 2). DNA sequencing was performed to confirm the deletions (Eurofins Genomics).

2.4. Complementation

Trans-complementation of the Δ*sleB* Δ*cwlJ* double mutant was performed with *sleB*, *cwlJ* or *sleB-ypeB* carried by the low-copy shuttle vector pHT315 (Arantes and Lereclus, 1991). The respective genes and associated regulatory sequences were amplified by PCR using primers A and D listed in Supplementary Table S1 and AccuPrim Taq DNA Polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions. The amplicons were cloned into pHT315 and the resulting constructs were used to transform electrocompetent *B. licheniformis* Δ*sleB* Δ*cwlJ* as described elsewhere (Mahillon et al., 1989). The presence of the correct plasmid construct was verified by PCR and sequencing.

2.5. Creation of fusion-constructs

Two different gene-fusions were constructed using alternative cloning techniques. The *cwlJ*^{P}*-sleB* construct, which consist of the predicted promoter region of *cwlJ* fused to *sleB* was created by PCR-amplification of the promoter region upstream of *cwlJ* (primer A and B, Table 1S) and of the entire *sleB* gene (primer C and D, Table 1S). To allow assembly of the PCR fragments, primers B and C contained complementary overlapping sequences. Finally, an additional PCR step was performed, using the upstream and downstream PCR fragments as template (primer A and D, Table 1S). The *sleB*^{N}*-cwlJ* construct, which encoded the *N*-terminal domain of *sleB* fused to full length *cwlJ*, was inserted into in pHT315 (Arantes and Lereclus, 1991) by using seamless cloning (Gibson et al., 2009; Perkel, 2014), using the primers listed in Table 1S.

2.6. Germination assays

Germination was monitored by measuring the decrease in optical density (OD_{600}) of spore suspensions using a 96-well microplate reader (Tecan Infinite M200, Grödig, Austria). Spores were first heat activated at 65 °C for 20 min, centrifuged for 3 min at 4500 × *g* at 4 °C and then resuspended in germination buffer (200 mM K-phosphate buffer, pH 7.2). The OD_{600} of the buffered spore suspension was adjusted to ~2.1 and 100 μL of 20 mM L-alanine (Sigma-Aldrich) was added to each well in a 96-well microplate plate (Falcon Flat Bottom, Becton Dickinson Labware, USA) containing 100 μL of buffered spore suspension. This gave an initial OD_{600} of approximately 1. Measurements were recorded at regular intervals of 2 min at 37 °C, and the plate was shaken for 10 s prior to each reading. The maximum germination rate (V_{max}) was calculated from the linear segment of the curves of OD changes (10–120 min) using Online DMFit (ComBase). DMFit (where "DM" stands for "dynamic modeling") fits curves where a linear phase is preceded by a lag phase and followed by a stationary phase (Baranyi and Roberts, 1994).

To examine spore viability, 500 μL of spore-suspensions (OD_{600} = 0.5) were heat treated at 80 °C for 30 min before serially diluted aliquots were plated on blood agar. The blood agar plates were then incubated for 17–18 h at 37 °C before counting colony forming units (CFUs).

DPA release was monitored by Tb-DPA fluorescence using 96-well flat bottom white microplates and the aforementioned Tecan Infinite M200 plate reader. *B. licheniformis* spores at an OD_{600} of ~0.1 were germinated at 37 °C in 150 μL of 100 mM K-phosphate buffer (pH 7.2) with 50 mM L-alanine as germinant. The spore suspensions also contained 250 μM terbium chloride. DPA release was monitored by real-time measurement of fluorescence emission at 545 nm with excitation at 270 nm, with Tb-DPA associated fluorescence being reported in arbitrary units (a.u.). Spore suspensions minus L-alanine were used as negative controls. Experiments were carried out in duplicate using three independent batches of spores.

2.7. Muropeptide analysis

Structural analysis of peptidoglycan associated with dormant *B. licheniformis* MW3 spores and fragments generated by *in vivo* CLE activity during germination was achieved using HPLC-MS protocols essentially as described previously (Christie et al., 2010). For germination experiments, heat shocked spores were suspended in 1 mL 200 mM potassium phosphate buffer, pH 7.2, supplemented with 20 mM L-alanine, at an OD_{600} of 60. Germinating spores were incubated at 37 °C for 90 min, after which samples were centrifuged (13,000 g for 1 min), and then the peptidoglycan-containing supernatants boiled for 3 min to inactivate residual CLE activity. Lyophilised samples were digested with mutanolysin and reduced with sodium borohydride prior to analysis (Christie et al., 2010). Identification of desalted muropeptide samples was achieved via MALDI mass spectrometry analysis with fragments generated by in-source-decay facilitating structural interpretation. A number of muropeptides, including those present only during germination, were subject to further HPLC analyses, where co-elution with established muropeptides was used to further validate

Table 2
Putative CLEs in the *B. licheniformis*.

Protein	Size (aa)	Function	Specificity	Identity to homologue in *B. subtilis*
SleB	321	Cell wall hydrolase with peptidoglycan binding domain	*N*-acetylmuramoyl-L-alanine amidase	70%
CwlJ	142	Cell wall hydrolase	*N*-acetylmuramoyl-L-alanine amidase	84%
CwlJ2 (YkvT)	199	Cell wall hydrolase	Amidase	50%
SleL (YaaH)	439	Glycosylhydrolase with LysM domains	*N*-acetylglucosaminidase	77%

**Bacillus subtilis* subsp. *subtilis* strain 168.*

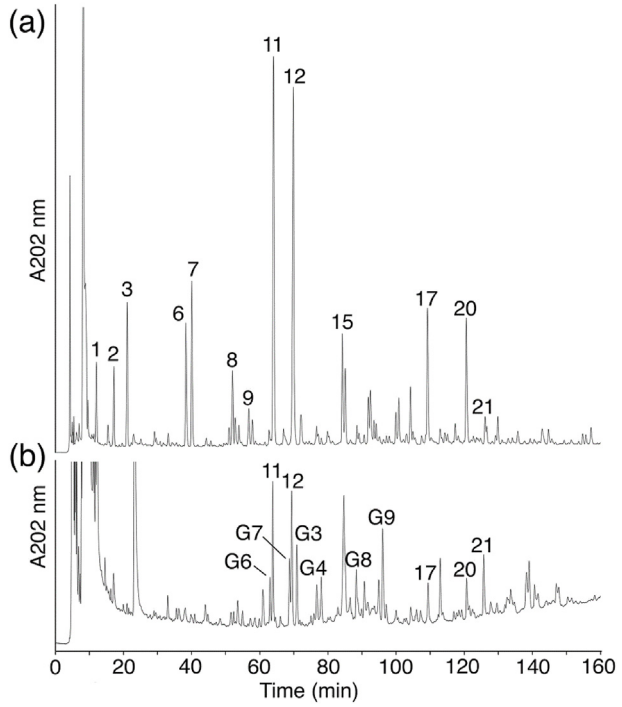


Fig. 1. Reverse phase HPLC profiles of mutanolysin-digested peptidoglycan associated with dormant (a), and germinating (b) *B. licheniformis* spores. Soluble peptidoglycan fragments were collected 90 min after initiation of spore germination in 200 mM potassium phosphate buffer, pH 7.2, supplemented with 20 mM L-alanine. Peptidoglycan from dormant spores and those present in the germination exudate were digested with mutanolysin and then reduced with sodium borohydride prior to HPLC separation. Peak identification was achieved by MALDI MS analysis; full descriptions are detailed in (Christie et al., 2010). Peaks identities: 1, DS-TriP; 2, DS-Ala; 3, DS-TP; 6, TSred-Ala; 7, TSred-TP; 8, DS-TriP x DS-TP; 9, DS-TP x DS-TP; 11, TS-TP; 12, TS-Ala; 15, TS-TP x DS-TP; 17, HS-Ala + Ala; 20, OS-Ac-Ala + Ala; 21, HS-Ala; G3, TS-TP; G4, TS-Ala; G6, TriS-TP; G7, TriS-Ala; G8, Anhydro-TS-TP; G9, Anhydro-TS-Ala. G-designated peaks are associated with CLE activity during spore germination.

proposed structures.

3. Results

3.1. Structural analysis of B. licheniformis spore peptidoglycan

Muropeptide analyses of peptidoglycan extracted from dormant *B. licheniformis* MW3 spores are indicative of a peptidoglycan structure that is very similar to that observed in other species of *Bacillus* (Foster and Johnstone, 1987; Popham et al., 1996). These analyses support a structurally distinct germ cell wall, comprising disaccharide monomers (muropeptides 1, 2 and 3) and a cortex comprised of predominantly tetrasaccharide-containing monomers (muropeptides 11 and 12) (Fig. 1a). Analysis of changes that occur to the spore peptidoglycan during germination proved challenging since germination appeared to proceed very slowly under the conditions employed. However, germination exudates collected 90 min after the initiation of germination contained sufficient peptidoglycan fragments to enable muropeptide analyses, which revealed lytic transglycoslyase (muropeptides G8 and G9), glucosaminindase (G6 and G7) and epimerase (G3 and G4) activity during *B. licheniformis* spore germination (Fig. 1b).

3.2. CLE genes in B. licheniformis

To search for the presence of CLE genes in the *B. licheniformis* DSM 13 (ATCC 14580) genome (NCBI:txid279010), CLE genes from *B. subtilis* (subsp. *subtilis* str. 168) were used as query sequences for BLAST searching (Rey et al., 2004; Veith et al., 2004). The searches resulted in the identification of four CLE gene homologues (Table 2). All four CLE proteins, deduced from their complementary DNA sequences, showed a high degree of identity (50–84%) to their respective homologues in *B. subtilis* throughout the entire sequences (Supplementary Fig. S1).

When comparing the upstream regions of *B. licheniformis* and *B. subtilis sleB, cwlJ* and *sleL* genes, potential σ^G promoter elements were detected in the −10 and −35 regions upstream of *sleB*, while potential σ^E promoter elements were recognized in the −10 and −35 regions upstream of *cwlJ* and *sleL* in the *B. licheniformis* genome (Table 3).

3.3. Expression of sleB, cwlJ, cwlJ2 and sleL at different time points during sporulation

Microscopy of *B. licheniformis* grown in sporulation medium revealed the first phase bright mature spores between 8 and 12 h (Fig. 2a). Quantitative Real Time-PCR was performed to determine the relative expression levels of *sleB*, *cwlJ, cwlJ2* and *sleL* with respect to *rpoB* in *B. licheniformis* (Fig. 2b). The *rpoB* gene encodes the RNA polymerase beta (β) subunit. Transcription of all putative CLE genes, apart from *cwlJ2*, was observed to increase markedly with respect to *rpoB* at or around the time of entry to sporulation (5 h post inoculation), with transcription of *cwlJ* and *sleL* being higher than *sleB* transcription early in sporulation (1 and 1.4 log higher expression levels respectively ($p < 0.01$)). From eight to 21 h post-inoculation, when approximately 50% of cells in the culture were observed to be forming spores, the transcription levels of *sleB, cwlJ* and *sleL* were in the same range as for *rpoB*. At all time points, the transcription level of *cwlJ2* was significantly lower than the expression of *sleB, cwlJ* and *sleL* (Fig. 2).

3.4. Germination of CLE null mutant spores

To examine the role of the putative cortex lytic enzymes in *B. licheniformis* spore germination, spores of in-frame *sleB, cwlJ, sleL* and *cwlJ2* deletion mutants were tested initially for their ability to germinate when suspended in buffer supplemented with 10 mM of L-alanine (Fig. 3). Spores lacking *sleB* showed a reduced germination rate compared to wild type spores and spores of the other single gene deletion mutants, with germination adjudged by OD_{600} and V_{max} being comparable to the negative control (MW3 spores suspended in buffer

Table 3
Comparison of the 5' region of *sleB*, *cwlJ* and *sleL* of *B. subtilis* and *B. licheniformis*.

Species/gene	Sequence 5′- 3′		Regulon	Bp upstream of ATG	Reference
	35	10			
B.s/sleB	GCGT**GTATA**AAATCTGCCTCGCTACA**AAAGATA**TG		σ^G	32	Moriyama et al. (1999)
B.l/sleB	AATA**GCTTT**CTTTCATGGGCAATATC**AAAGATA**			72	
B.s/cwlJ	CGTC**ATCACT**TCTGAAGTAATG**AAATATGAT**		σ^E	27	Ishikawa et al. (1998)
B.l/cwlJ	CGTC**ATCACC**TAAATGAAACA**GAATATGAT**			31	
B.s/sleL	ATAA**ACATGAT**CAGCGCTTTTCTTT**CATACATT**GATA		σ^E	38	Kodama et al. (1999)
B.l/sleL	TAGG**CTAACT**TATAGTTAAAGG**AATATAAA**ATCTG			77	

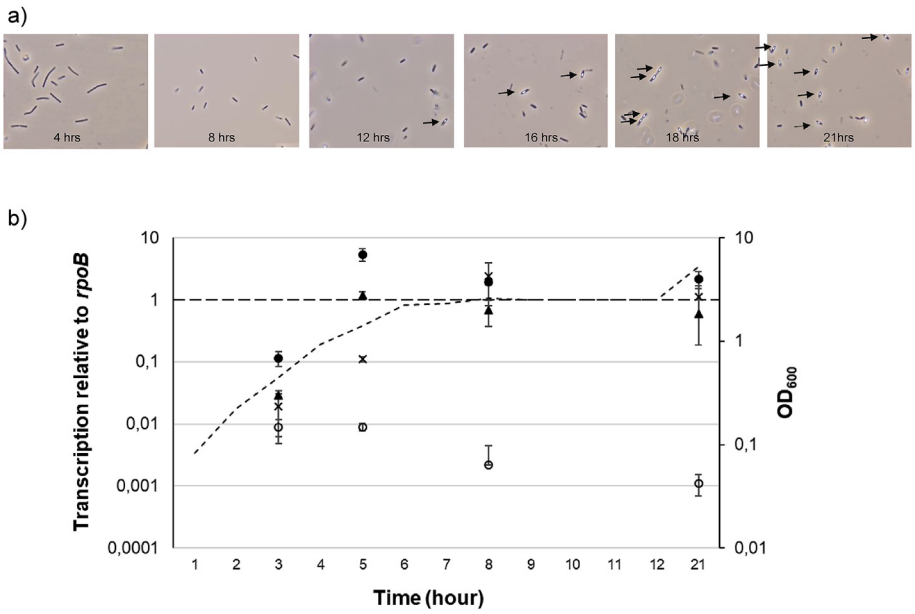


Fig. 2. Phase contrast microscopy images of *B. licheniformis* incubated in sporulation medium for 4, 6, 8, 12 16, 18 and 21 h (a). Mature phase-bright spores are indicated by arrows. Transcription level of *sleB* (x), *cwlJ* (▲), *cwlJ2* (○) and *sleL* (●) relative to *rpoB* determined by qRT-PCR during 21 h of growth of *B. licheniformis* MW3 (b). The dotted line represents the bacterial growth measured by increase in OD_{600}. At 21 h there were about 50% spores in the cultures as observed by phase contrast microscopy. Whiskers represent standard deviation of three independent experiments.

without alanine) (Fig. 3a and e). V_{max} for the *sleB* mutant and the negative control were 0.06 ± 0.001 and 0.05 ± 0.003, respectively, with respective reductions in OD_{600} of 26% ± 10% and 24% ± 8%. Δ*cwlJ* spores displayed a slight delay in OD_{600} loss compared to wild type spores over the first 40 min after alanine exposure (Fig. 3a) but OD_{600} loss observed after 120 min, and V_{max} (1.15 ± 0.10), were comparable to those of wild type spores (Fig. 3e). Spores lacking either *cwlJ2* or *sleL* germinated in a similar manner to Δ*cwlJ* spores, but in both cases the final absorbance loss was less than observed in wild type spores, although not significantly so. As expected, Δ*sleB* Δ*cwlJ* double mutant spores showed a germination efficiency that was comparable to the Δ*sleB* single mutant and negative control spores (Fig. 3b and e). In addition to OD_{600} measurements, the release of CaDPA during germination was analysed for some of the CLE mutants (Fig. 3f). The Δ*cwlJ* mutant released CaDPA as the wild type, while the Δ*sleB* mutant, confirming the results achieved by monitoring loss of OD600, was completely blocked in CaDPA release. Unexpectedly, however, the Δ*cwlJ* Δ*sleB* double mutant released CaDPA near the wild type level.

Similarly, when either *cwlJ2* or *sleL* was deleted in the Δ*sleB* Δ*cwlJ* background it did not change germination efficiency compared to the double mutant background (Fig. 3b). Collectively these results indicate that SleB plays the most pronounced role in alanine-induced germination of *B. licheniformis* spores, with CwlJ, CwlJ2 and SleL, appearing to have lesser roles in germinative efficiency at the population level.

3.5. Germination of complementing mutants

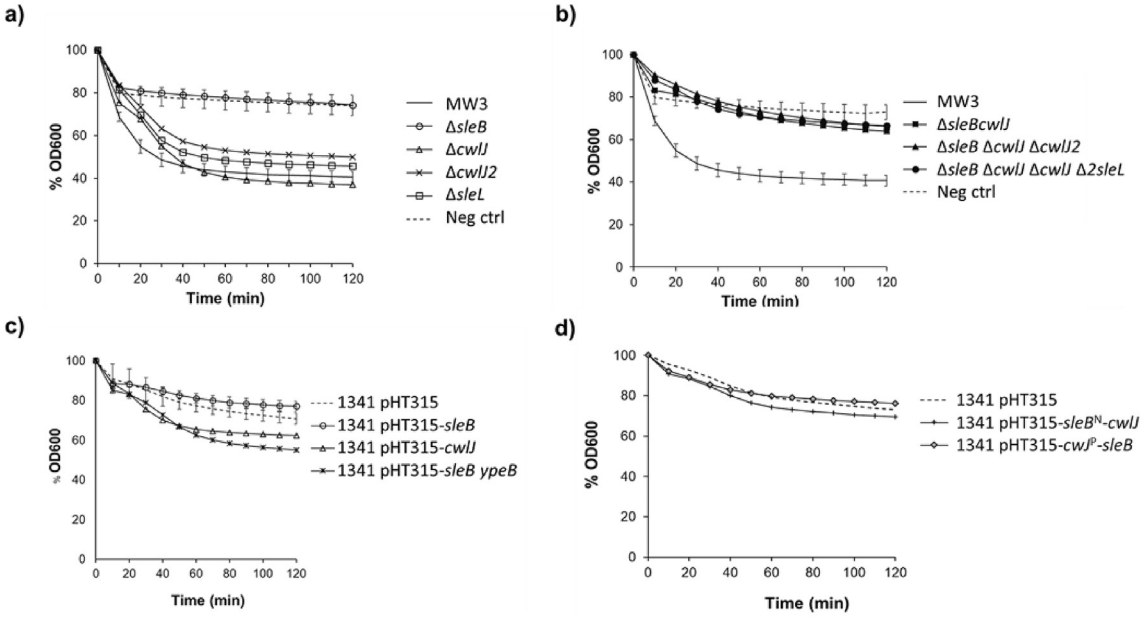
To verify the role of the different CLE genes in spore germination trans-complementation experiments were performed in the Δ*sleB* Δ*cwlJ* background. Δ*sleB* Δ*cwlJ* spores carrying the empty pHT315 vector showed a reduced drop in OD_{600} (7%) compared to parental Δ*sleB* Δ*cwlJ* spores; additionally, the viability was reduced to close to 0, indicating that the plasmid had a negative effect on germination and outgrowth (Figs. 3 and 4). Accordingly, in order to avoid bias due to the presence or absence of plasmid, germination properties of the complemented spores were compared to those of Δ*sleB* Δ*cwlJ* pHT315 spores. Trans-complementation with *sleB* alone had a slightly negative effect on germination efficiency with L-alanine compared to Δ*sleB* Δ*cwlJ* pHT315 spores (Fig. 3c and e). In contrast, complementation with both *sleB* and *ypeB* conferred a more efficient germinative response compared to spores of the background strain (Fig. 3c and e), with V_{max} increasing from −0.17 ± 0.04 (*sleB*) to −0.55 ± 0.10 (*sleB ypeB*). A positive effect on spore germination was also observed when the Δ*sleB* Δ*cwlJ* mutant was complemented with *cwlJ* alone, which changed the V_{max} from −0.19 ± 0.16 to −0.67 ± 0.35 (Fig. 3e). These results indicate a necessity of co-transcription of *sleB-ypeB* for an optimal initiation of germination.

3.6. Germination of spores expressing CLE fusion constructs

Since CwlJ is homologous to the catalytic domain of SleB we sought to determine whether a fusion of the 5' region of *sleB,* which encodes a PG-binding domain, and the entire *cwlJ* gene could restore CLE activity in the Δ*sleB* Δ*cwlJ* background. The fusion construct was cloned into pHT315, positioned to be under the control of the *sleB* promoter, and introduced to the Δ*sleB* Δ*cwlJ* strain. However, the L-alanine induced germinative response of spores carrying this genetic construct was comparable to Δ*sleB* Δ*cwlJ* pHT315 spores (Fig. 3d and e and 4), indicating limited, if any, functionality. In a second experiment, Δ*sleB* Δ*cwlJ* spores carrying plasmid borne *sleB* placed under control of the *cwlJ* promoter sequence were prepared to examine if mother-cell expression (as opposed to fore-spore) and presumed localisation in the spore influences SleB functionality. Again, germination efficiency of the resultant spores in response to L-alanine was comparable to the germination response of Δ*sleB* Δ*cwlJ* pHT315 spores, indicating either a lack of functionality or presence of the protein in the spore (Fig. 3e).

3.7. Spore viability

To examine the effect of SleB, CwlJ, SleL and CwlJ2 on spore viability, both wild type and CLE mutant spores were incubated at 80 °C (to kill any vegetative cells present) before plating on blood agar plates. Spores of the Δ*sleB* mutant showed a significantly reduced viability (2-log) compared to wild type spores, and indeed spores of the respective Δ*cwlJ,* Δ*sleL* and Δ*cwlJ2* null mutant strains, none of which were associated with significant defects in viability (Fig. 4). Deletion of *cwlJ* in the *sleB* background reduced viability further, with spores of the Δ*sleB* Δ*cwlJ* strain being reduced by 6 logs compared to wild type spores. Further deletion of *cwlJ2* and *sleL* in the Δ*sleB* Δ*cwlJ* mutant background did not significantly alter spore viability (Fig. 4). Trans-complementation of the Δ*sleB* Δ*cwlJ* mutant with *sleB* alone restored the spore viability to wild type levels (Fig. 4). However, the rate of OD_{600} loss was still reduced compared to spores of the wild type strain. To achieve wild type levels of OD_{600} loss, the presence of both *sleB* and *ypeB* on the



e)

Strain	Description	V_{max} 100 mM L-ala (Drop in OD min^{-1})	Drop in OD_{600} after 120 min (%)
MW3	*B.licheniformis* DSM13 Δ*hsdR1* Δ*hsdR2*	-0.97 ± 0.10 (0.07 ± 0.01)*	59 ± 3 (30 ± 2)*
NVH-1331	Δ*sleB*	-0.06 ± 0.01	26 ± 10
NVH-1333	Δ*cwlJ*	-1.15 ± 0.10	63 ± 3
NVH-1341	Δ*sleB* Δ*cwlJ*	-0.41 ± 0.04	36 ± 4
NVH-1372	Δ*sleB* Δ*cwlJ pHT315-sleB*^{N}-*cwlJ*	-0.36 ± 0.08	31 ± 5
NVH-1374	Δ*sleB* Δ*cwlJ pHT315-sleB*	-0.17 ± 0.04	23 ± 3
NVH-1375	Δ*sleB* Δ*cwlJ pHT315-cwlJ*	-0.67 ± 0.49	38 ± 12
NVH-1379	Δ*sleB* Δ*cwlJ pHT315-cwlJ*^{P}-*sleB*	-0.27 ± 0.13	24 ± 2
NVH-1384	Δ*cwlJ* Δ*sleB* Δ*cwlJ2*	-0.46 ± 0.08	34 ± 5
NVH-1388	Δ*cwlJ2*	-1.14 ± 0.52	50 ± 6
NVH-1430	Δ*sleL*	-1.09 ± 0.09	54 ± 3
NVH-1432	Δ*sleB* Δ*cwlJ* Δ*cwlJ2* Δ*sleL*	-0.45 ± 0.15	33 ± 6
NVH-1469	Δ*sleB* Δ*cwlJ pHT315*	-0.33 ± 0.14	27 ± 5
NVH-1470	Δ*sleB* Δ*cwlJ pHT315-sleB ypeB*	-0.55 ± 0.10	45 ± 4

*Negative control (no germinant added)

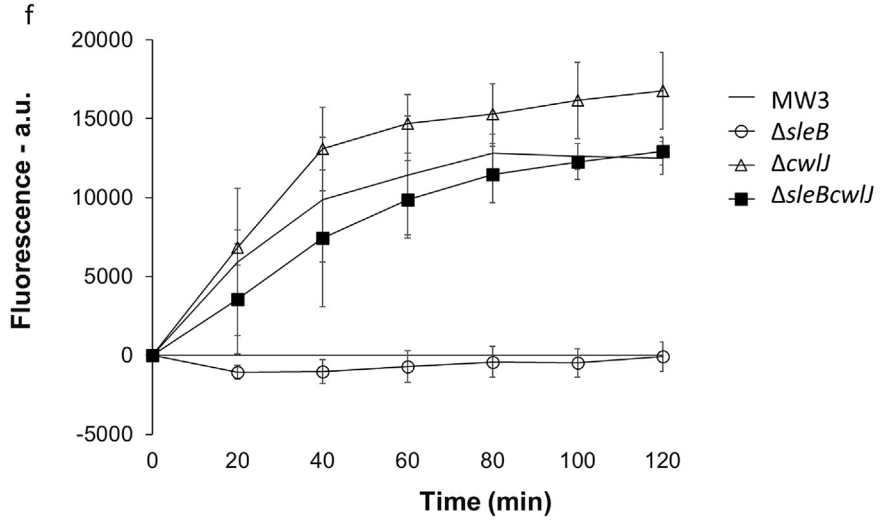


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Fig. 3. Spore germination of *sleB*, *cwlJ*, *sleL* and *cwlJ2* deletion mutants and mutants complemented with their corresponding wild type genes. All mutants were derived from the MW3 strain. Germination was measured by decrease in OD_{600} over a period of 120 min after addition of germinant. Germination of wild type (MW3) spores and of single mutant spores (a). Germination of wild type spores and of double, triple and quadruple mutant spores (b). Trans-complementation of deleted genes with wild type copies carried on the multicopy plasmid pHT315 (c). Trans-complementation in Δ*sleB* Δ*cwlJ* background by *sleB* behind the *cwlJ* promoter (pHT315-*cwlJ*^{P}-*sleB*), and of the PG binding encoding part of *sleB* fused to *cwlJ* (pHT315-*sleB*^{N}-*cwlJ*) (d). V_{max} of drop of OD_{600} (Drop in OD_{600} min^{-1}) for curves shown in a-d (e). CaDPA release of CLE mutant spores germinated with 50 mM L-alanine in presence of 250 μM $TbCl_3$ monitored by Tb-DPA fluorescence (given in arbitrary units [a.u.]) (f). Standard deviation of three independent spore preparations are indicated.

plasmid was necessary (Fig. 3b and c). This indicates that co-transcription of *sleB-ypeB* is necessary for an optimal initial germination response. However, *sleB* transcription alone is sufficient for spore viability and outgrowth in rich medium. When the Δ*sleB* Δ*cwlJ* mutant was complemented with *cwlJ* both loss of OD_{600} and viability were restored (Fig. 3b and c, Fig. 4). Trans-complementation with *sleB* placed under control of the *cwlJ* promoter was observed to moderately, but significantly, increase the viability of Δ*sleB* Δ*cwlJ* spores. A small but not significant increase in viability was observed also for spores with the *sleB*^{N}-*cwlJ* fusion construct in the double mutant background (Fig. 4).

4. Discussion

The major purpose of this work was to characterize cortex lytic enzyme activity associated with the germination of *B. licheniformis* spores, which, despite its significance as a spoilage organism, had not yet been studied. The bulk of the results are largely consistent with observations made previously in other species of *Bacillus*. These include spore peptidoglycan structure, which was shown for *B. licheniformis* to have a similar composition to that observed in other *Bacillus* spores (Atrih et al., 1998; Dowd et al., 2008; Christie et al., 2010). Subsequent muropeptide analyses conducted with germinating spores revealed the presence of enzymatic activities also observed previously in other spore formers. These include lytic transglycosylase activity, which based on the relative abundance of anhydromuropeptides present in germination exudates appears to be the major lytic activity during *B. licheniformis* spore germination. Muropeptides derived from *N*-acetylglucosaminidase activity were also detected, although at reduced abundance compared to lytic transglycosylase products. The anhydromuropeptides G8 and G9 are almost certainly SleB derived products, given that the hydrolytic bond specificity of orthologues of this enzyme has been characterized previously (Ustok et al., 2014). Unfortunately, however, the poor germinative efficiency of the *sleB* null mutant strain precluded successful muropeptide analyses of germination exudates aimed at validating this suggestion. Candidate enzymes associated with the observed *N*-acetylglucosaminidase activity include SleL, characterized as such in other *Bacillus* species (Lambert and Popham, 2008; Ustok et al., 2014), or orthologous enzymes such as YdhD (Chen et al., 2000). Intriguingly, despite SleL unambiguously being associated with *N*-acetylglucosaminidase activity, the presence of epimerase-derived muropeptides G3 and G4 has been shown to require an intact copy of *sleL*, at least in *B. subtilis* and in *B. megaterium* (Atrih et al. 1998, 1999; Christie et al., 2010). While not tested in this work, it seems likely that this will be the case in *B. licheniformis* also, although the significance of this non-lytic modification to the peptidoglycan during germination has not been determined, nor the enzyme(s) directly responsible. Future work in this area should aim to clarify this situation.

In addition to *sleB* and *sleL*, two additional putative CLE genes – *cwlJ* and *cwlJ2* (*ykvT*) - were found in the genome of *B. licheniformis* ATCC 14580. Quantitative RT-PCR analyses indicate that in *B. licheniformis, cwlJ* is more highly expressed than *sleB* (as is *sleL*). The *cwlJ2* gene is expressed at very low levels, and actually decreases during sporulation, suggesting – as in *B. subtilis* (Chirakkal et al., 2002) - that the protein may not be involved in sporulation and/or be component of the spore. Indeed, the expected gradual decline in transcription levels of CLE genes, when the cells are nearing completion of the sporulation process, was not observed in this study. This is probably due to the asynchronous sporulation observed in these cultures, where only ~50% of the population had sporulated after 21 h (Fig. 2b).

The most notable result from investigating the role of the different CLE gene homologues in *B. licheniformis* concerns the relative importance of SleB and CwlJ in spore germination. These proteins are often described as being semi-redundant in the sense that efficient spore germination will proceed in the absence of either protein but not both. In *B. licheniformis*, however, deletion of *cwlJ* results in a minor germination defect as adjudged by absorbance loss, with no impact on spore viability on rich culture medium. In contrast, deletion of *sleB* results in a severe germination defect, with viability on rich medium being reduced

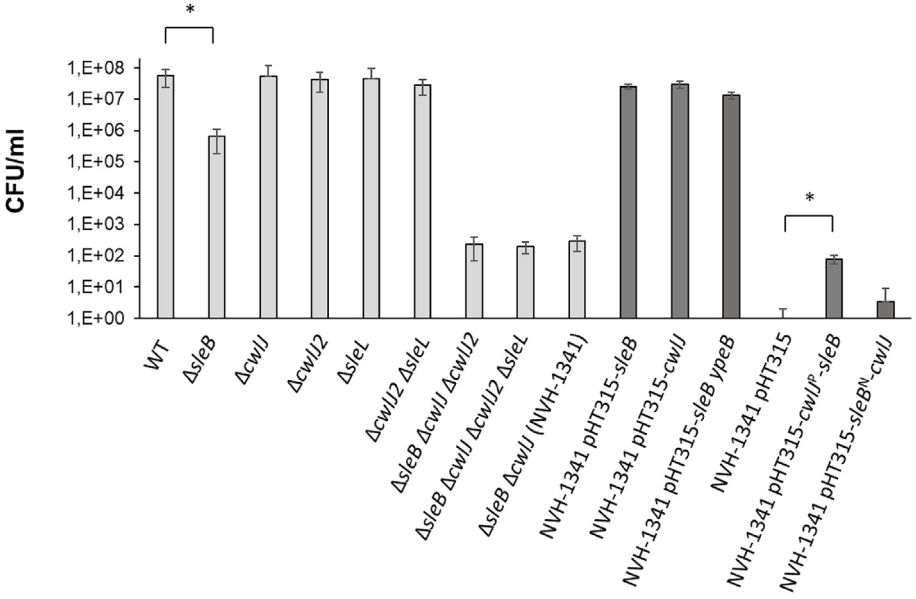

Fig. 4. Colony-forming efficiency of CLE mutants. The colony-forming efficiency was measured as CFU/mL of a given spore stock after incubation for 17–18 h at 37 °C on blood agar plates after prior incubation for 30 min at 80 °C. The data presented are mean of three individual experiments with SD indicated. Asterisks represent statistical differences from pairwise comparisons using two-tailed paired Student t tests.*; $P < 0.05$.

by two logs compared to wild type spores. Levels of absorbance loss for spores suspended in alanine-containing buffer are also significantly reduced, being comparable to spores suspended in buffer that does not contain germinant (microscopy observations indicate that the initial loss in absorbance of these spores is due to spore clumping as opposed to release of CaDPA). Similarly, Δ*sleB* spores do not release detectable amounts of CaDPA for at least 2 h after the addition of germinant (Fig. 3f). In contrast, *cwlJ* null mutant spores release CaDPA at levels that are commensurate with wild type spores, which is in accordance with losses in OD_{600} associated with these spores. Unexpectedly, however, *sleB cwlJ* double mutant spores exposed to germinant appear to lose absorbance at an intermediate rate compared to Δ*sleB* and Δ*cwlJ* spores. This observation is reinforced by CaDPA measurements, which revealed that *sleB cwlJ* double mutant spores release CaDPA at levels commensurate with wild type spores when exposed to germinant. It seems therefore that the absence of SleB prevents the release of CaDPA, at least for up to 2 h after addition of germinant, but that the additional absence of CwlJ in this background somehow permits rapid CaDPA release. This apparent interplay between CLEs on the release of CaDPA from germinating spores has not been observed in other species of *Bacillus* and further investigation will be required to elucidate the mechanisms involved.

Deletion of *sleB* in *B. subtilis, B. megaterium* and *B. anthracis* also results in reduced spore viability (0.5 logs for *B. subtilis* and *B. megaterium* and 0.1 log for *B. anthracis*) (Ishikawa et al., 1998; Heffron et al., 2009; Setlow et al., 2009; Christie et al., 2010), and in impaired absorbance loss, but considerably less than observed in *B. licheniformis*. It seems, therefore, that CwlJ can only weakly compensate for the loss of SleB in *B. licheniformis*, and seemingly to a lower degree than evident in other species. Equally, it has been shown previously that germination in populations of spores is not particularly synchronized, and that the time before CaDPA is released may vary significantly between spores within a population (Kong et al., 2010; Wang et al., 2011). Accordingly, this must be taken into consideration when OD_{600} loss, spore viability and outgrowth data are being evaluated. The compensatory effect of CwlJ is evident, however, in the *sleB* background, with the viability of double mutant spores being reduced by 6 logs compared to 2 logs when *sleB* alone has been deleted. This pronounced reduction in spore viability after deletion of both *cwlJ* and *sleB*, compared to individual deletions of either *cwlJ* or *sleB*, is observed also in other *Bacillus* species (Ishikawa et al., 1998; Heffron et al., 2011). Spores bearing additional deletions of *sleL* and or *cwlJ2* exhibit similar germination properties to the parental Δ*sleB* Δ*cwlJ* strain, indicating that the SleL and CwlJ2 proteins have minimal influence on the germinative efficiency of *B. licheniformis* spores.

Other notable results in this work concern outputs from complementation type experiments conducted in the Δ*sleB* Δ*cwlJ* background. Plasmid borne *sleB* restored full viability to Δ*sleB* Δ*cwlJ* spores, for example, but did not restore efficient absorbance loss in alanine-containing buffer. The latter was restored, however, when double mutant spores were complemented with plasmid borne *sleB* and its bicistronic partner *ypeB*. Considering that the *sleB* deletion is in-frame, and shouldn't affect *ypeB* transcription, these data may indicate that *sleB* and *ypeB* have to be co-transcribed for optimal levels of SleB, and or YpeB, in the spore. The observation that plasmid borne *cwlJ* restored full viability to Δ*sleB* Δ*cwlJ* spores, together with moderate absorbance loss in response to L-alanine, was also unexpected given the aforementioned reliance on SleB for efficient germinative responses in *B. licheniformis*. One explanation for this may be that ectopic expression of *cwlJ* results in an increased abundance of CwlJ in the spore compensating for the absence of SleB (pHT315 derived plasmids have a copy number of 10–15 copies per cell (Arantes and Lereclus, 1991)). The placing of *sleB* or *cwlJ* under control of inducible promoters may permit the impact of varying concentrations of these proteins on cortex hydrolysis and CaDPA release to be examined in the future.

Finally, in an attempt to gain insight to CLE function in spores, experiments aimed at examining elements of interchangeability between SleB and CwlJ were conducted. First, the influence of mother cell versus forespore expression of SleB was examined by introducing a construct with *sleB* being driven by the *cwlJ* promoter ($cwlJ^{P}$-*sleB*) into the Δ*sleB* Δ*cwlJ* strain. The second experiment involved a domain swap, where a construct encoding the PG binding domain of SleB and the catalytic domain of CwlJ ($sleB^{N}$-*cwlJ*) was introduced into Δ*sleB* Δ*cwlJ* spores. Germination of the resultant spores, in both cases, was poor, with only mother cell expressed SleB conferring a slight ($P > 0.05$) increase in viability with respect to the parental *sleB cwlJ* spores.

5. Conclusion

Cortex lytic enzymes involved in depolymerisation of cortical peptidoglycan in *B. licheniformis* spores are similar to those reported previously in other species of *Bacillus*. The major enzymatic activity detected during germination is that of a lytic transglycosylase, almost certainly SleB, as adjudged by muropeptide and mutational analyses. The SleB protein appears to have a more prominent role in the germination of *B. licheniformis* spores than in spores of other *Bacillus* species, CwlJ (at normal expression levels) alone being inadequate for efficient spore germination and colony formation on nutrient medium.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103259.

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