RESEARCH LETTER

Characterization of a spore-specific protein of the *Bacillus cereus* group

Cecilie From¹, Menno van der Voort²,³, Tjakko Abeë² & Per Einar Granum¹

¹Department of Food Safety and Infection Biology, Section for Food Safety, Norwegian School of Veterinary Science, Oslo, Norway; ²Laboratory of Food Microbiology, Wageningen University and Research Centre, Wageningen, The Netherlands; and ³Laboratory of Phytopathology, Wageningen University and Research Centre, Wageningen, The Netherlands

Correspondence: Per Einar Granum, Department of Food Safety and Infection Biology, Section for Food Safety, Norwegian School of Veterinary Science, Ullevålsveien 72, PO Box 8146 Dep., 0033 Oslo, Norway. Tel.: +47 22 96 48 45; fax: +47 22 96 48 50; e-mail: PerEinar.Granum@nvh.no

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Abstract

*Bc1245* is a monocistronic chromosomal gene of *Bacillus cereus* ATCC 14579 encoding a putative protein of 143 amino acids identified in this study to have a spore-related function in *B. cereus*. *Bc1245* is highly conserved in the genome of members of the *B. cereus* group, indicating an important function of the gene in this group of bacteria. Quantitative PCR revealed that *bc1245* is transcribed late in sporulation (upon formation of phase-bright spores) and at the same time as the mother cell–specific transcription factor σK. The σK regulon includes structural components of the spore (such as coat proteins), and it is therefore plausible that *bc1245* might encode a structural outer spore protein. This was confirmed by detection of BC1245 in exosporium extracts from *B. cereus* by immunoblotting against BC1245 antiserum.

Introduction

*Bacillus* encompasses species capable of forming highly resistant dormant endospores as a response to environmental stress such as nutrient deprivation (Setlow & Johnson, 2007, and references therein). When receiving a specific signal (nutrient or nonnutrient derived), spores are able to come back to life as vegetative cells in an irreversible process called germination and subsequent outgrowth (Moir *et al.*, 2002; Setlow, 2003). Endospore formation is a tightly controlled differentiation process involving activation of a serial of alternative spore or mother cell-specific sigma factors in a sequential order which coordinate the expression of mRNA involved in spore development (Kroos & Yu, 2000). At the end of sporulation, the mother cell lyses and a mature metabolically inactive spore with a highly ordered structure is released. The innermost part of the spore is the dehydrated core that contains large amounts of Ca²⁺-dipicolinic acid (Ca²⁺-DPA) and DNA protected from degradation by tight binding to small acid soluble proteins (Setlow, 1995). Outside the core is a specialized peptidoglycan cortex (Popham, 2002; Dowd *et al.*, 2008) surrounded by a complex protein shell called the coat that consists of more than 50 polypeptides assembled in several distinct layers that vary between species (Driks, 1999, 2002; Henriques & Moran, 2000; Kim *et al.*, 2006). The exosporium is a loosely attached balloon-like structure encasing the outermost surface of spores of some species including the food pathogen *Bacillus cereus*, the causative agent of anthrax *Bacillus anthracis* as well as nonpathogens such as *Bacillus megaterium* and *Bacillus subtilis* (Vary, 1994; La Duc *et al.*, 2004). It consists of an outer layer of hair-like projections and one or more inner basal layers with a crystal-like appearance (Gerhardt & Ribi, 1964). Another crystalline layer (a parasporal layer) located within the interspace between the coat and the exosporium has recently been described together with a molecular 3-D model of the spore surface architecture (Kailas *et al.*, 2011). Although it has been postulated that the exosporium is important in interaction with host organisms and for attachment of spores to surfaces such as certain eukaryotic cell types (Basu *et al.*, 2007), the precise function of the exosporium is still to be elucidated (Ball *et al.*, 2008).
In later years, a number of proteins making up the exosporium have been identified and characterized mainly in *B. cereus* and *B. anthracis* (Steichen *et al.*, 2003; Todd *et al.*, 2003; Redmond *et al.*, 2004; Giorno *et al.*, 2009). The collagen-like glycoprotein BcA is a major component of the external hair-like nap and the best-characterized exosporium protein (Sylvestre *et al.*, 2002; Steichen *et al.*, 2003). Another collagen-like glycoprotein, BcB, is found to have an important role in exosporium assembly (Thompson *et al.*, 2007; Thompson & Stewart, 2008). Also ExsFA/BxpB and ExsFB needed for the anchoring of BcA to the basal layer (Sylvestre *et al.*, 2005; Tan *et al.*, 2011) and ExsY required for the complete assembly of the exosporium (Boydston *et al.*, 2006) were recently identified among others. It is assumed that the exosporium harbors a number of other structural proteins (Kailas *et al.*, 2011; Thompson *et al.*, 2011a, b) and more loosely attached proteins such as enzymes that may reduce the sensitivity of spores to germinants have been described (Todd *et al.*, 2003).

In this paper, we report on the identification and initial characterization of a novel spore-specific protein in *B. cereus*. Our current findings suggest that the protein is part of an outer spore structure, most likely the exosporium or the interspace between the exosporium and the coat.

**Materials and methods**

**Strains and plasmids used in this study**

The bacterial strains used in this study were the *B. cereus* type strain ATCC 14579 (Frankland & Frankland, 1887; Ivanova *et al.*, 2003) and *B. subtilis* B252 (From *et al.*, 2005). To create a bc1245 deletion mutant in *B. cereus* ATCC 14579, a shuttle vector modified from pMAD B252 (From *et al.*, 2004) with a spectinomycin-resistant cassette in the restriction site SalI (Fagerlund, 2007) was used.

**In silico genome analysis**

Sequence information was obtained from the NCBI bacterial genome database (http://www.ncbi.nlm.nih.gov/guide) or the ERGO database (Overbeek *et al.*, 2003). Comparative genomic analyses of bc1245 were performed on selected members of the *B. cereus* group [ *B. cereus* ATCC 14579 (GenBank: NC004722), *B. cereus* ATCC 10987 (GenBank: NC003909), *B. cereus* AH187 (GenBank: CP001177), *Bacillus thuringiensis* YBT-020 (GenBank: CP002508), *B. anthracis* str. Ames (GenBank: AE016879), *Bacillus weihenstephanensis* KBAB4 (GenBank: NC010184), *B. mycoderis* DSM 2048 (GenBank: CM000742) and *B. pseudomycoderis* DSM12442 (GenBank: CM000745)] to investigate whether bc1245 is conserved. Putative σ-binding sites for the bc1245 promoter were predicted by analyzing the 500-bp upstream region of bc1245 with DBTBS release 5 (Sierro *et al.*, 2008). To search for functional motifs, the amino acid sequence of BC1245 was submitted to ScanProSite, (http://www.expasy.ch/prosite; Bairoch *et al.*, 1997).

**RNA isolation and quantitative PCR**

Quantitative PCR experiments were performed as described previously (van der Voort *et al.*, 2010), and primers were designed by use of Primer 3 (Rozen & Skaletsky, 2000) for sigH, sigE, sigF, sigG, sigK, bc1245 and zcDNA (Table 1) using the chromosomal DNA sequence of *B. cereus* ATCC 14579 as a template. PCR on genomic DNA was used to check primer efficiency (results not shown). RNA was isolated from two independent cultures withdrawn at different stages of sporulation of *B. cereus* ATCC 14579 grown in maltose sporulation medium (MSM) as described earlier (van der Voort *et al.*, 2010). cDNA synthesis was performed with ~500 ng of total RNA and a mix of relevant reverse primers as described previously (van Schaik *et al.*, 2007). Quantitative PCR was performed with 5 μM of each of the primer pairs listed in Table 1 using an ABI Prism 7700 with SYBR green technology (PE Applied Biosystems, Nieuwekerk a/d Ijssel, the Netherlands) as described previously (van Schaik *et al.*, 2005). By comparing expression of the chosen genes with that of the reference 16S rRNA gene (zcDNA) levels, relative expression values were obtained with the REST-MCS program using the Pair Wise Fixed Reallocation Randomization Test (Pfaffl *et al.*, 2002).

**Construction of a bc1245-mutant strain**

To inactivate bc1245, the gene was replaced by the spectinomycin-resistant gene (*spc*) through a double crossover

**Table 1. Primers used for quantitative PCR**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>sigHF</td>
<td>GGTGCGCATCGGAAGACAT</td>
</tr>
<tr>
<td>sigHR</td>
<td>TCAGCAATGCGGATTGAGAAGA</td>
</tr>
<tr>
<td>sigEF</td>
<td>TGCCACCACCCATGTAACAA</td>
</tr>
<tr>
<td>sigFR</td>
<td>ATGGTACGATTGGGCTCATTA</td>
</tr>
<tr>
<td>sigG</td>
<td>CGCGATGATGGATCAGTGAAGA</td>
</tr>
<tr>
<td>sigGR</td>
<td>TGGGACCCCTCGGAATTTCTT</td>
</tr>
<tr>
<td>sigKF</td>
<td>GCAAGTCGGAAGATACAGCGAAGAG</td>
</tr>
<tr>
<td>sigER</td>
<td>TACAGGGAATGGGCAATCATC</td>
</tr>
<tr>
<td>sigKR</td>
<td>TACAGGGAATGGATCAATC</td>
</tr>
<tr>
<td>Bc1245F</td>
<td>GCGATGATGGATCAGTGAAGA</td>
</tr>
<tr>
<td>Bc1245R</td>
<td>ATGGGAAATGGGCAATCATC</td>
</tr>
</tbody>
</table>
event using the pMAD vector and the protocol according to Arnaud et al. (2004). The regions located directly upstream and downstream from bc1245 were amplified from B. cereus ATCC 14579 using the following primer pairs, respectively: TCAAGAATTCGATTTTGCCCTCT ACTC/TAAGAATTCGATAAGATGAAAA and ATAAAGATCCCTCTATACCAAGACTGT/GAATTGACCGAAATTGATAAGACAGAT (restriction sites underlined). The fragments were inserted into the pMAD vector on either side of spc, producing the pMADΔbc1245 plasmid and the directions of the inserts were verified by PCR. The pMADΔbc1245 plasmid was introduced into B. cereus ATCC 14579 by electroporation (Masson et al., 1989), and the bc1245 deletion mutant was obtained as described by Arnaud et al. (2004). Replacement of bc1245 by spc was verified by sequencing of PCR products using the primer pairs TCAAGCATATTCAGTATTTCCATAAAAC, respectively.

Sporulation

Bacteria were sporulated either using the MSM (van der Voort et al., 2010) or a chemically defined sporulation medium (de Vries et al., 2004). Sporulation was continued until ≥ 90% phase bright spores were observed by phase contrast microscopy (~1–2 days after incubation in sporulation medium). Spores were harvested by centrifugation for 10 min at 8000 g at 4 °C, prior to resuspension in 10 mL cold autoclaved MQ with 0.1% Tween for MSM spores or 10 mM K-phosphate buffer pH 7.2 for spores made in chemically defined medium. Spores were washed by centrifugation and resuspension in MQ with 0.1% Tween or 10 mM K-phosphate buffer pH 7.2 a total of ten times. The resulting spore crops contained <10% germinated spores (observed as phase-dark spores by phase-contrast microscopy) and were stored refrigerated in MQ (0.1% Tween) or 10 mM K-phosphate buffer pH 7.2.

Germination and outgrowth of wild-type and mutant B. cereus spores

Germination assays were performed as described earlier (Hornstra et al., 2005) by monitoring the reduction in absorbance at A600 as spores turn from phase bright to phase dark at 30 °C in a 96-well microplate in a plate reader (Tecan Infinite M200, Grödig, Austria). Heat-activated (70 °C, 15 min) and non-heat-activated spores prepared in MSM were adjusted to an initial A600 nm of ~2 (Shimadzu UV-VIS 160A; Shimadzu Europa GMBH) in germination buffer (final concentration in the assay 10 mM Tris pH 7.4, 10 mM NaCl) prior to addition of germinant. Germinants tested were 100 and 1 mM L-alanine, 10 and 1 mM inosine, a combination of 100 or 1 mM L-alanine and 10 or 1 mM inosine, 1 mM cysteine, 1 mM threonine and 1 mM glutamine.

Outgrowth was monitored for non-heat-activated spores prepared in chemically defined sporulation medium by absorbance readings at 30 °C for 24 h as described previously. Germination/outgrowth medium was 1/2× Brain Heart Infusion (BHI) broth 10 mM Tris 10 mM NaCl pH 7.4. Spore germination in Ca2+-DPA (final concentration ~50 mM DPA ~50 mM CaCl2) of spores prepared in chemically defined sporulation medium (initial A600 nm of ~1) was performed as described earlier (Vepachedu & Setlow, 2007), and germination was monitored by absorbance readings at 30 °C for 1.5 h as described previously.

Spore surface hydrophobicity and heat resistance

Spore surface hydrophobicity was measured as described by (Rosenberg et al., 1980) as follows: spores were washed once and dissolved in 1 mL 10 mM K-phosphate buffer pH 7.2 to A600 nm of ~0.6–0.4. 50 μL of N-hexadecane was added to the spore sample and vortexed for 1 min prior to incubation for 10 min at room temperature. Absorbance was measured for the watery phase of the sample, and percentage of hydrophobicity was given as A600 nm initial – A600 nm after N-hexadecane addition/A600 nm initial. Heat resistance was investigated by plate counting of spore suspensions (A600 nm ~0.5) of wild-type B. cereus ATCC 14579 and bcΔ1245 on LB agar plates after heat treatment in a water bath at 90 °C for 1, 3, 10 and 30 min and incubation at 37 °C overnight.

Exosporium isolation, SDS-PAGE and immunoblotting

Chemical extraction of exosporium was as follows: 1 mL of a spore suspension (~107 spores mL−1) was centrifuged at 16 100 g for 3 min, washed once in 1× phosphate-buffered saline pH 7.4 (Gibco/Invitrogen) and resuspended in 100 μL SDS-8 M urea sample buffer (Thompson et al., 2011a, b). The spores were boiled in SDS-8 M urea sample buffer for 10 min in a water bath, the sample was centrifuged and 20 μL of supernatant was used in consequent SDS-PAGE gel electrophoresis using the XCell SureLockTM Mini-Cell system (Invitrogen). Extracted spore proteins were separated on size on a 12% NuPAGE® Bis-Tris Gel (Invitrogen) run in 1× MOPS SDS Running Buffer (Invitrogen) at 200 volt for 45 min. Proteins were transferred to a nitrocellulose filter.
(0.45 μm; Bio-Rad), and immunoblotting was performed using 1× NuPAGE Transfer Buffer (Invitrogen) according to the manufacturer’s protocol. Polyclonal antibody against BC1245 was used to detect BC1245 on immunoblots in a dilution of 1 : 500 with biotin-conjugated goat anti-rabbit IgG (Invitrogen) in a dilution of 1 : 3000 as the secondary antibody. Immunoreactive proteins were detected as described earlier (Lindbäck et al., 2004) using a complex of streptavidin and biotinylated alkaline phosphatase (1 : 3000) before development with a NBT/BCIP solution (Bio-Rad). Anti-BC1245 antiserum was prepared commercially (BioGenes GmbH, Berlin, Germany) in rabbit following immunization with a synthetic peptide epitope derived from an amino acid sequence from the N-terminus of BC1245 (position 9–22: LPDEPQEP-KEPKPA). A cysteine residue on the N-terminus was added to enable the direct conjugation to the protein carrier. The molecular mass of the proteins was estimated using SeeBlue® Plus2 Pre-Stained Standard (Invitrogen). The experiment was repeated at least twice and on individual spore batches.

**Results**

### In silico and expression analysis

In *B. cereus* ATCC 14579, *bc1245* is a monocistronic chromosomal gene (GenBank: NP831029) encoding a 143 aa putative protein of unknown function with an estimated molecular weight of 15108 Da. Comparative genomic analysis of the gene and encoded amino acid sequence of *bc1245* in members of the *B. cereus* group (*B. cereus, B. anthracis, B. thuringiensis, B. weihenstephanensis* and *B. mycoides*) revealed that *bc1245* is highly conserved in this group of spore-forming bacteria, with nucleotide identity scores ranging between 81% and 98% (Table 2). The *B. pseudomyoides* gene was most distant from *bc1245* with 66% of nucleotide identity. The sequence is not found in the genome of spore-forming bacteria outside the *B. cereus* group (data not shown). Analyzing the 500-bp upstream region of *bc1245* identified two hypothetical σ^K-dependent promotor-binding sites, 223- and 178-bp upstream of *bc1245* (Table 3 and Fig. 1). A ProSite motif search revealed that BC1245 contains a short, conserved amino acid signature (DTITVTA) resembling a TonB-box starting 81 aa from the N-terminus (Fig. 1). As in *silico* analysis showed that *bc1245* transcription is putatively under control of a hypothetical σ^K-dependent promotor (Table 3 and Fig. 1), transcription was studied in relation to sporulation-related sigma factors encoding genes. Quantitative PCR showed expression of *sigH, sigE,* and *sigF* to decline after 13 h of incubation, expression of *sigG* and *sigK* remained high until 17 h of incubation. Moreover, *bc1245* is transcribed late in sporulation, and especially, expression was observed from 13 h until 17 h of incubation (upon formation of phase-bright spores), simultaneously with high expression of *sigG* and *sigK* (Fig. 2).

**No apparent phenotype revealed after inactivation of bc1245**

No difference in sporulation in MSM and a chemically defined medium was observed between wild-type *B. cereus* ATCC 14579 and a *bcA1245* deletion mutant. Both wild-type and mutant spores appeared the same when compared using phase-contrast microscopy (data not shown). No difference in heat stability or hydrophobic properties when compared to wild-type spores was detected. Both wild-type *B. cereus* and the mutant germinated efficiently (> 99% phase-dark spores as observed by phase-contrast microscopy after 1.5-h germination) in 100 and 1 mM l-alanine, 10 and 1 mM inosine, a combination of 100 or 1 mM l-alanine and 10 or 1 mM inosine, 1 mM cysteine and 50 mM Ca^{2+}-DPA. Both strains germinated less efficiently in 1 mM threonine and 1 mM glutamine (~ 50% phase-dark spores after 1.5-h germination). Outgrowth of the wild-type and *bcA1245*-mutant spores were followed both spectrophotometrically in a plate reader and by video filming (Olympus Bx51,

### Table 2. Comparison of the *bc1245* nucleotide and encoded amino acid sequence

<table>
<thead>
<tr>
<th>Species</th>
<th>Nucleotide identity (%)</th>
<th>Protein identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em> ATCC 14579</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>B. cereus</em> ATCC 10987</td>
<td>82</td>
<td>77</td>
</tr>
<tr>
<td><em>B. cereus</em> AH187</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> YBT-020</td>
<td>98</td>
<td>98*</td>
</tr>
<tr>
<td><em>B. anthracis</em> str. Ames</td>
<td>98</td>
<td>98*</td>
</tr>
<tr>
<td><em>B. weihenstephanensis</em> KBAB4</td>
<td>81</td>
<td>76</td>
</tr>
<tr>
<td><em>B. mycoides</em> DSM 2048</td>
<td>84</td>
<td>77</td>
</tr>
<tr>
<td><em>B. pseudomyoides</em> DSM 12442</td>
<td>66</td>
<td>56*</td>
</tr>
</tbody>
</table>

*Sequence in these species is not annotated to be a protein.*

### Table 3. Hypothetical σ^K-dependent promotor-binding sites for *bc1245* in Bacillus cereus ATCC 14579 identified by DBTBS release 5 (Sierra et al., 2008)

<table>
<thead>
<tr>
<th>Hypothetical σ^K-dependent promotor-binding site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (−178 bp)*</td>
<td>TACCATCCTTCTTTTTACATACCTT</td>
</tr>
<tr>
<td>P2 (−223 bp)*</td>
<td>TACATACCTTACGTTTTCCATATTTT</td>
</tr>
</tbody>
</table>

*Promoter-binding site 1 (P1) starts 178-bp upstream of *bc1245.*

†Promoter-binding site 2 (P2) starts 223-bp upstream of *bc1245.*
spores in BHI with germinants (100 mM L-alanine, 10 mM inosine) on a microscopic slide in phase contrast (100×). No apparent differences in wild-type and mutant spore outgrowth were observed (data not shown).

BC1245 is a component of the exosporium

As bc1245 has a putative σ^K-dependent promotor and is transcribed late in sporulation, we wanted to investigate whether BC1245 is a component of an outer structure of the spore such as the exosporium. Anti-BC1245 antiserum raised in rabbit indeed detected BC1245 in a fraction of exosporium extracted from wild-type spores. BC1245 was not detected in extracted samples from bc1245^-mutant or B. subtilis spores (which lack both bc1245 and an exosporium; Fig. 3).

Discussion

Identification and characterization of several structural proteins of both the inner basal layer(s) and the external
projections of the exosporium has in recent years increased our knowledge on this poorly understood component of the bacterial spore (Charlton et al., 1999; Sylvestre et al., 2002; Steichen et al., 2003; Todd et al., 2003; Redmond et al., 2004; Fazzini et al., 2010; Terry et al., 2011; Thompson et al., 2011a, b). The current study identified BC1245 as a spore-specific protein. Bc1245 is highly conserved in members of the B. cereus group (B. anthracis, B. cereus, B. thuringiensis and B. weihenstephanensis) supportive of an important function of the gene (and possibly its gene product) in this group of bacteria. Members of the B. cereus group are known to have an exosporium as the outermost part of their spores, and as bc1245 was present in this group of bacteria while other Bacilli species such as B. subtilis lack the gene, we wanted to investigate whether bc1245 encode an exosporium protein. In silico analysis indicated that the bc1245 promotor was under control of the mother cell–specific sigma factor K (σ^K), which regulon in B. subtilis includes a series of genes encoding outer spore structural components such as coat proteins (Errington, 1993; Haldenwang, 1995). Real-time PCR revealed that bc1245 is transcribed late in sporulation (at the onset of phase-bright spores) and expressed at the same time as high expression of sigG and sigK. Although expression is declining, sigE and sigF are also expressed in the time frame of bc1245 expression. Further studies on expression of bc1245 in sigma factor-mutant strains and determination of the transcription start will determine the sigma factor-regulating expression of bc1245. The combination, however, of the prediction of a sigma factor K-dependent promotor and simultaneous expression with sigK make it plausible that bc1245 might encode a structural outer spore protein in the σ^K regulon. A recent study describing a novel exosporium protein BetA used the finding of putative σ^K-directed promotor elements as a search criterium when looking for genes encoding exosporium proteins in B. anthracis (Thompson et al., 2011a, b). Also exosporium proteins BcD and BxP are preceded by a consensus sequence for a promotor recognized by σ^K (Sylvestre et al., 2002; Steichen et al., 2003). Unfortunately, we do not yet know the function of BC1245 as a bc1245 mutant was unaltered in spore heat resistance, hydrophobicity, germination and outgrowth capacity when compared with wild-type B. cereus. Further characterization of the mutant spore would be valuable, for example, visualization of the outer spore surface by different microscopic techniques such as electron cryomicroscopy or atomic force microscopy as described by Kailas et al. (2011). To further identify the localization of BC1245 in the spore immunoblotting using BC1245 antisera targeting a commercially synthesized epitope of the BC124 protein was performed on an SDS-8 M urea exosporium extract from the type strain of B. cereus ATCC 14579. As BC1245 was detected in an extract using the SDS-8 M urea extraction protocol, it is likely that BC1245 is an exosporium protein or a protein localized in the inter-space between the exosporium and the underlying coat layer of the spore. However, we cannot exclude the possibility that coat proteins are also extracted by this method and that Bc1245 antisera reacted with such a coat protein. Notably, BC1245 contains a short, conserved region (DTITVTA) starting 81 aa from the N-terminus that is identical to the TonB-box of the TonB-dependent outer membrane transporter FhuA of Escherichia coli (Table 1 in Postle & Larsen, 2007). TonB-dependent membrane transporters are common in Gram-negative bacteria and have a conserved motif, the Ton-box (Lundrigan & Kadner, 1986; Schramm et al., 1987) that interacts with the TonB-protein in the inner membrane complex during active transport of essential micro-nutrients across the outer and inner (plasma) membrane (Wiener, 2005; Shultis et al., 2006). To our understanding, TonB-dependent membrane transporters have not been described in Gram-positive bacteria, and hence, the role of a TonB-box in BC1245 is unclear.

In conclusion, we have identified and partly characterized a novel spore-specific protein BC1245. The function and precise localization of BC1245 within the exosporium remains to be elucidated.

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References


