Collagen-Like Glycoprotein BclS Is Involved in the Formation of Filamentous Structures of the *Lysinibacillus sphaericus* Exosporium

Ni Zhao,^{a,b} Yong Ge,^a Tingyu Shi,^a Xiaomin Hu,^a Zhiming Yuan^a

Key Laboratory of Applied and Environmental Microbiology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China^a; University of the Chinese Academy of Sciences, Beijing, China^b

Lysinibacillus sphaericus produces mosquitocidal binary toxins (Bin toxins) deposited within a balloon-like exosporium during sporulation. Unlike *Bacillus cereus* group strains, the exosporium of *L. sphaericus* is usually devoid of the hair-like nap, an external filamentous structure formed by a collagen-like protein, BclA. In this study, a new collagen-like exosporium protein encoded by BspH_0411 (BclS) from *L. sphaericus* C3-41 was characterized. Thin-section electron microscopy revealed that deletion of *bclS* resulted in the loss of the filamentous structures that attach to the exosporium basal layer and spread through the interspace of spores. *In vivo* visualization of BclS-green fluorescent protein (GFP)/mCherry fusion proteins revealed a dynamic pattern of fluorescence that encased the spore from the mother cell-distal (MCD) pole of the forespore, and the BclS-GFP fusions were found to be located in the interspace of the spore, as confirmed by three-dimensional (3D) superresolution fluorescence microscopy. Further studies demonstrated that the *bclS* mutant spores were more sensitive to wet-heat treatment and germinated at a lower rate than wild-type spores and that these phenotypes were significantly restored in the *bclS*-complemented strain. These results suggested novel roles of collagen-like protein in exosporium assembly and spore germination, providing a hint for a further understanding of the genetic basis of the high level of persistence of Bin toxins in nature.

The genus *Bacillus* includes a diverse collection of Gram-positive, aerobic, and rod-shaped bacteria that form a spore in response to starvation and harsh environments (1). Formation of the spores results from two primitive cell differentiation processes involving two different cell types, the larger mother cell and the smaller forespore. The forespore is engulfed by the mother cell and develops into a mature spore, which is surrounded by a thick layer of modified peptidoglycan (called the cortex) and a multi-layered protein shell (called the coat). In some *Bacillus* species, including *B. subtilis*, *B. licheniformis*, and *B. macerans*, the coat is the outermost layer of the spore (2), whereas in many members of the *B. cereus* group, the spore is further covered by a loose-fitting and balloon-like exosporium composed of a paracrystalline basal layer and a hair-like nap layer (3). In order for the exosporium to be able to assemble around the spores, there should be some connections between the exosporium and the coat, a region referred to as the interspace (4). As the outermost contact surface between the spores and the environment, the exosporium has many functions, such as in interactions with soil (5), in the display of surface antigens (6), in initiation of cell infection (7, 8), and as a semipermeable barrier to environmental insults (9).

Thus far, several *Bacillus* collagen-like (Bcl) proteins have been identified in *B. cereus* group strains, including BclA, BclB, BclC, BclD, and BclE (10), of which only BclA has been demonstrated to be a major component of the hair-like nap layer, a filamentous structure protruding from the surface of the exosporium (11). BclA is a highly glycosylated protein that contains an internal collagen-like region (CLR) of GXY repeats linked to multiple copies of a tetrasaccharide (12). It has been shown that the length of the BclA CLR is responsible for the variation in filament length of the hair-like nap (13) and that the assembly of the BclA filaments is dependent on the basal layer protein BxpB (14). The initial assembly of the BclA protein was oriented toward the mother cell compartment, away from the pole of the mother cell (15), consistent with the exosporium assembly of *B. cereus* and *B. anthracis* (16,

17). In addition to BclA, BclB and BclE contain a conserved N-terminal domain that is responsible for assembly into the exosporium (10), but only BclA and BclB were verified experimentally to be integrated into the exosporium (15). In contrast, BclC and BclD lack the consensus targeting sequence; whether they are associated with the exosporium remains to be further investigated (10). Most recently, three BclA homologs (BclA1, BclA2, and BclA3) were identified in *Clostridium difficile* (18), and the glycosylation of BclA3 was dependent on a glycosyltransferase gene located upstream of the *bclA3* gene (19).

Lysinibacillus sphaericus (formerly *Bacillus sphaericus*) is a Gram-positive, aerobic, and spore-forming bacterium that is commonly isolated from soils (20). Some strains produce mosquitocidal proteins and thus have been widely used as biocontrol agents for disease-transmitting mosquitoes (21). The most potent mosquitocidal component of *L. sphaericus* is the binary toxins (Bin toxins), which are produced during sporulation in the form of a parasporal crystal (22). Unlike *B. thuringiensis*, whose insecticidal proteins are usually released from the spore during sporulation, the Bin toxins of *L. sphaericus* are enveloped by the exosporium, which is proposed to play a role in protecting the crystal from degradation (23). Furthermore, spores of *L. sphaericus* are able to germinate in the larval midgut, propagate vegetatively, and develop into new spores (24, 25). These particular properties endue *L. sphaericus* strains with greater persistence in the field than *B. thuringiensis* (26, 27).

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Address correspondence to Zhiming Yuan, yzm@wh.iov.cn.

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
<i>Escherichia coli</i>		
BL21	Expression host	Laboratory stock
JM109	Cloning host	Laboratory stock
<i>L. sphaericus</i>		
C3-41	Wild-type <i>L. sphaericus</i> strain	36
$\Delta bclS$ mutant	<i>bclS::kan</i>	This study
<i>bclS</i> -complemented mutant	<i>bclS::kan amyE::(P_{bclS}-bclS spc)</i>	This study
BSpBUG	$\Delta bclS$ mutant with plasmid pBUG	This study
BSpBUSG	$\Delta bclS$ mutant with plasmid pBUSG	This study
BSpBUSM	$\Delta bclS$ mutant with plasmid pBUSM	This study
Plasmids		
pGIC086	Source of the kanamycin resistance gene (<i>kan</i>)	22
pRN5101	Shuttle vector of <i>E. coli</i> and <i>L. sphaericus</i> with a temp-sensitive replicon	37
pRN5101S	<i>bclS</i> inactivation vector	This study
pMarB333	Source of the spectinomycin resistance gene (<i>spc</i>)	29
pRN-CbclS	<i>bclS</i> complementation vector	This study
pBU4	High-copy-no. shuttle vector of <i>E. coli</i> and <i>L. sphaericus</i>	38
pHT315- <i>gfp</i>	Source of the green fluorescent protein gene (<i>gfp</i>)	39
pRSET-B- <i>mCherry</i>	Source of the <i>mCherry</i> gene	40
pBUG	pBU4 carrying <i>P_{bclS}-gfp</i>	This study
pBUSG	pBU4 carrying <i>P_{bclS}-bclS-gfp</i>	This study
pBUSM	pBU4 carrying <i>P_{bclS}-bclS-mCherry</i>	This study
pET28a	IPTG-inducible expression vector allowing fusion of a C-terminal His ₆ tag to the target protein	Novagen
pET28aS	BclS ₉₈₂₋₁₀₂₃ expression vector	This study

Although the exosporium has been studied extensively in the *B. cereus* group, relatively little is known about the proteins forming the *L. sphaericus* exosporium, which is usually devoid of the hair-like nap (28). In this study, a collagen-like exosporium protein of *L. sphaericus* C3-41, named BclS, was demonstrated to be essential for the formation of filamentous structures in the interspace. Further investigations revealed that BclS played a role in heat resistance, spore germination, and outgrowth. We believe that this information is of importance for understanding the diversified functions of collagen-like proteins in exosporium assembly as well as the persistence mechanism of the Bin toxins in nature.

MATERIALS AND METHODS

General methods. Strains and plasmids used in this study are listed in Table 1, and primers are listed in Table 2. *Escherichia coli* was grown in Luria-Bertani broth (LB) at 37°C, and *L. sphaericus* was grown at 30°C in MBS medium, composed of 0.68% KH₂PO₄, 0.03% MgSO₄ · 7H₂O, 0.002% MnSO₄, 0.002% Fe₂(SO₄)₃, 0.002% ZnSO₄ · 7H₂O, 0.002% CaCl₂, 1% tryptone, and 0.2% yeast extract (pH 7.2). Antibiotic concentrations were as follows: ampicillin at 100 µg/ml and kanamycin at 50 µg/ml for *E. coli* and kanamycin at 10 µg/ml, spectinomycin at 100 mg/ml, erythromycin at 5 µg/ml, and tetracycline at 5 µg/ml for *L. sphaericus*.

Spore preparation. Fresh cultures of *L. sphaericus* cells grown overnight were prepared by agitation at 30°C in LB, and aliquots of 800 µl were plated onto 15-cm solid MBS plates containing 2% agar, followed by incubation at 30°C until the preparations showed >95% brightly refractile dormant spores, as examined by phase-contrast microscopy.

Construction of *L. sphaericus* mutants and BclS-green fluorescent protein (GFP)/mCherry fusions. For disruption of *bclS* from C3-41, a 936-bp upstream fragment (0411-A) and an 876-bp downstream fragment (0411-B) of *bclS* were amplified from the C3-41 genome by using primers SL-F/SL-R and SR-F/SR-R, respectively. Primer pair Kan-F/

Kan-R was used to amplify a 1,321-bp kanamycin resistance gene (*kan*) from plasmid pGIC086. Fragment 0411-A, *kan*, and fragment 0411-B were digested with SalI/NheI, NheI/KpnI, and KpnI/HindIII, respectively, and cloned between the SalI and HindIII sites in temperature-sensitive plasmid pRN5101. The resulting plasmid, pRN5101S, was introduced

TABLE 2 Primers used in this study

Primer	Nucleotide sequence (5'-3') ^a
SL-F	ACGCGTCGACCTGGCACTACGGGTGTAACGG
SL-R	CTAGCTAGCCAATCCCAGCAACGCCAGTG
SR-F	CGGGGTACCTTACTGAAGGATTCTCTGCC
SR-R	CCCAAGCTTAAACAGTGCTACAGGATT
Kan-F	CCCGCTAGCGAACCATTGAGGTGATAGG
Kan-R	CCCGGTACCGGTACTAAAACAATTCATCCAG
amyLA-F	CCCGTCGACGAATAATGGGCAAATTCAGTGG
amyLA-R	CCCGGATCCAGAAAGCGTCTTGTCTTGACGA
amyRA-F	CCCGAATTCATTTACAAAAGAAATAGCA
amyRA-R	CCCGCTAGCGGATGTGATTTACCAAAGTCT
PS-F	CGCGGATCCGTCTACCTCTCTATCAATTCACAG
PS-CR	CCCGGTACCCTAAGAAATTCGGTGACATGACCAAACG
spcR-F	CCCGGTACCAGAGTTGGTAGCTCTTGATCCG
spcR-R	CCCGAATTCCTTTAAAAGTAAGCACCTGTTATTC
GFP-F	ACGCGTCGACTAGATGAGTAAAGGAGAAGAACTTTTCAC
GFP-R	AAGTGCAGTTATTTGTATAGTTCATCCATGCCA
Pro2-R	ACGCGTCGACAGTAGCCCCAGATAGCCCCA
PS-R	ACGCGTCGACAGAAATTCGGTGACATGACCAAACG
mCherry-F	ACGCGTCGACATGGTGAGCAAGGGCGAGGAG
mCherry-R	CCCAAGCTTCTACTTGTACAGCTCGTCCATGCC
SP10-F	CCGGAATTCGGACCTGCGTTTACTGAAGGATTC
SP10-R	CGCAAGCTTAGTAGTTGGATTGAAAGCAGGTGTTG

^a Restriction enzyme recognition sites are underlined.

into C3-41 by electroporation according to methods described previously (29). The allele replacement mutant ($\Delta bclS$) was screened for resistance to kanamycin and sensitivity to erythromycin and confirmed by PCR analyses.

The *bclS*-complemented mutant was obtained by insertion of a wild-type copy of *P_{bclS}-bclS* into the chromosome of the $\Delta bclS$ mutant at the *amyE* locus (Bsph_1204). An 863-bp upstream fragment (*amy*-A) and a 938-bp downstream fragment (*amy*-B) of the *amyE* gene were amplified from the C3-41 genome by using primers *amy*LA-F/*amy*LA-R and *amy*RA-F/*amy*RA-R, respectively. A 3,888-bp fragment containing the intact *bclS* gene and its native promoter (*P_{bclS}*) was amplified with primer pair PS-F/PS-CR, and a 1,214-bp spectinomycin resistance gene (*spc*) was amplified from plasmid pMarB333 by using primer pair SpcR-F/SpcR-R. The *amy*-A fragment, *P_{bclS}-bclS*, *spc*, and the *amy*-B fragment were digested with *Sall*/*Bam*HI, *Bam*HI/*Kpn*I, *Kpn*I/*Eco*RI, and *Eco*RI/*Nhe*I, respectively, and cloned between the *Sall* and *Nhe*I sites in pRN5101 to generate plasmid pRN-*CbclS*. Following electroporation into the $\Delta bclS$ mutant, colonies with spectinomycin and kanamycin resistance but lacking erythromycin resistance were selected and confirmed by PCR analysis, and the mutant was designated the *bclS*-complemented strain (Table 1).

The *gfp* gene was amplified from pHT315-*gfp* by using primer pair GFP-F/GFP-R, and the 495-bp *P_{bclS}* region was amplified from the C3-41 genome by using primer pair PS-F/Pro2-R. The two purified PCR fragments were digested with *Sall*/*Pst*I and *Bam*HI/*Sall*, respectively, and cloned into the *Bam*HI and *Pst*I sites of plasmid pBU4 (Table 1), generating plasmid pBUG. A 3,885-bp *P_{bclS}-bclS* fragment was amplified with primer pair PS-F/PS-R, digested with *Bam*HI/*Sall*, and cloned in frame upstream of *gfp* between the equivalent sites of pBUG, yielding plasmid pBUSG. An *mCherry* gene was amplified from plasmid pRSET-B-*mCherry* with primer pair *mCherry*-F/*mCherry*-R, digested with *Sall*/*Hind*III, and cloned between the equivalent sites of pBUSG, yielding plasmid pBUSM. Plasmids pBUG, pBUSG, and pBUSM were introduced into the $\Delta bclS$ mutant by electroporation.

Protein overexpression. Plasmid pET28aS was constructed for overproduction of a deletion derivative of the BclS protein. The *bclS* gene fragment encoding residues 982 to 1023 was amplified with primer pair SP10-F/SP10-R, digested with *Eco*RI/*Hind*III, and cloned into similarly digested pET28a. Plasmid pET28aS was transformed into *E. coli* BL21(DE3), and the truncated protein of BclS (BclS_{982–1023}) was overexpressed by inducing an exponential-phase culture with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) at 30°C for 6 h. Cells were collected, washed and resuspended in phosphate-buffered saline (PBS), and sonicated for 30 min. BclS_{982–1023} was purified from the insoluble fraction by electrophoresis on a 15% SDS-PAGE gel, and the correct-size band was excised and used to immunize mice, yielding BclS antiserum.

Western blotting and glycoprotein characterization. For Western blotting, spore proteins were extracted according to a method described previously (11). The samples were separated on an 8% SDS-PAGE gel, blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P[®]; Millipore), and probed with mouse anti-BclS serum (1:3,000) followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Millipore).

After separation by 8% SDS-PAGE and transfer onto PVDF membranes, the glycoproteins were detected by using a glycoprotein detection kit (Amersham) according to the manufacturer's instructions. The protein bands were visualized by using the BeyoECL Plus reagent (Beyotime, China).

Electron and fluorescence microscopy. For transmission electron microscopy (EM), mature spores were fixed and embedded in Epon 812 resin and subjected to ultrathin sectioning. Thin-section electron microscopy was performed as described previously (28), and the samples were photographed by using a Tecnai G² 20 transmission electron microscope at 200 kV. For fluorescence microscopy, 3 μ l of the spores was spotted onto a glass slide and examined by phase-contrast and fluorescence microscopy, using an Olympus BX51TFR microscope equipped with a

mercury lamp device (U-RFL-T; Olympus). The three-dimensional (3D) superresolution images of individual structures were taken by using a DeltaVision OMX fluorescence microscope supplied with a digital camera (Applied Precision).

Measurement of heat resistance. Spores of the wild-type, $\Delta bclS$, and *bclS*-complemented strains were washed three times with ice-cold distilled water by repeated centrifugation. Approximately 10⁸ spores were resuspended in 1 mM phosphate buffer (pH 7.4) and treated at 65°C, 70°C, 75°C, and 80°C for 20 min. Aliquots at appropriate dilutions were plated onto MBS plates and incubated for 18 h at 30°C. Colony counts were compared to those of untreated control samples. The experiments were performed in duplicate with three different preparations of wild-type and mutant spores, and statistical significance was analyzed by Student's *t* test.

Germination and outgrowth assays. Spores of the wild-type, $\Delta bclS$, and *bclS*-complemented strains were washed 10 times with ice-cold distilled water to remove cell debris. The germination assay was performed according to a method described previously (30). Briefly, spores were resuspended and heat activated at 45°C for 25 min in ultrapure water, followed by resuspension in MBS medium to an optical density at 600 nm (OD₆₀₀) of 1.0. The OD₆₀₀ values were determined at 1-min intervals for 1 h at 30°C by using a Synergy HT plate reader (BioTek, USA). Spores were then collected, and the phase-bright and phase-dark spores were counted by phase-contrast microscopy. The outgrowth assay was performed based on a method described previously (31), and the OD₆₀₀ of the spore suspension was measured at 20-min intervals at 30°C.

RESULTS AND DISCUSSION

Identification of BclS. Spores of *L. sphaericus* C3-41 are devoid of the hair-like nap layer composed of the BclA protein, the best-characterized Bcl glycoprotein of *B. cereus* group organisms. Attempts were thus made to search Bcl homologs against the whole genome sequences of *L. sphaericus* C3-41. The results showed that Bsph_0411, encoding a protein of 1,142 amino acid residues, harbored a hydrophobic CLR at residues 88 to 984 and showed 18% amino acid similarity to BclA (BAS1130) and 41% similarity to BclE (BAS4623) of *B. anthracis* Sterne. Bsph_0411 (BclS) had a strong preponderance of GXT triplets in the CLR, but the dominant triplet was GAT, rather than GPT in BclA and GST in BclE. The variation in the number of CLR GXY triplets (299 in Bsph_0411, 76 in BclA, and 158 in BclE) seemed to be responsible for the differences in sequence similarity. These analyses suggested that Bsph_0411 encodes a new bacterial collagen-like protein named BclS.

Further analyses revealed a homolog of the *B. anthracis* glycosyltransferase gene (BAS1131) located upstream of the *bclS* gene, Bsph_0413, which may be responsible for the transfer of carbohydrate components to the BclS protein. Western blotting with anti-BclS antiserum showed that a single protein band for BclS was detected in wild-type but not in $\Delta bclS$ spores (Fig. 1A), and the molecular mass of detected BclS (>300 kDa) was much higher than that estimated from polypeptide analysis (~98 kDa). The Amersham ECL glycoprotein detection module (Amersham Biosciences) was therefore used to test for glycosylated residues, and a single protein band corresponding to the BclS protein was detected in spore extracts of the wild type, whereas no obvious band was observed in spore extracts of the $\Delta bclS$ strain (Fig. 1B). These results suggested that BclS is a glycoprotein.

Exosporium morphology of wild-type, $\Delta bclS$, and *bclS*-complemented strains. Negative-stain EM showed that 80 to 90% of the wild-type or the *bclS* mutant spores retained an intact exosporium after washing 10 times (>300 spores inspected per sample),

FIG 1 Immunoblotting and glycan detection. Equal amounts of protein samples from wild-type (WT) or $\Delta bclS$ spores were separated by 8% SDS-PAGE and analyzed by Western blotting using the BclS polyclonal antibody (A), and glycans were detected by using a glycoprotein detection kit (B).

indicating that the *bclS* mutation had no significant effect on the stability of the exosporium. Longitudinal-section EM revealed that the disruption of *bclS* resulted in the loss of filamentous structures (Fig. 2C), which attached to the exosporium basal layer and spread through the interspace (Fig. 2A). Cross-section EM showed more electron-dense deposits visible in the interspace of wild-type spores (Fig. 2B) than in $\Delta bclS$ spores (Fig. 2D). Complementation of the *bclS* mutation with one copy of the *bclS* gene

significantly restored the wild-type pattern of filamentous structures, as examined by both longitudinal-section (Fig. 2E) and cross-section (Fig. 2F) EM. These results indicated that BclS is involved in the formation of filamentous structures of the *L. sphaericus* interspace. The filament-forming properties of BclS were consistent with the composition of collagen-like triplets, in which a large number of GXY repeats is always a structural indicator of collagen-like triple helices even in phylogenetically distant proteins (32).

Subcellular localization of BclS. To visualize the subcellular localization of BclS, the expression of the BclS-GFP fusion protein in $\Delta bclS$ cells (BSpBUSG) (Table 1) was monitored at different time points before and during sporulation. As shown in Fig. 3A and B, no fluorescence was observed during vegetative growth. Three hours after entering stationary phase (T_3), terminally swelling cells were detected, and the cells displayed diffuse and cytoplasmic fluorescence (Fig. 3C and D). Two hours later (T_5), the forespore became semirefractile, and the GFP fluorescence appeared as a cap-like structure at the mother cell-distal (MCD) pole of the forespore (Fig. 3E and F). At T_6 , the fluorescence completely engulfed the whole forespore (Fig. 3G and H). Phase brightness approached a maximum at T_8 , and GFP fluorescence at the

FIG 2 Electron micrographs showing longitudinal sections (right) and cross sections (left) of spores of various *L. sphaericus* strains. (A and B) Wild type; (C and D) $\Delta bclS$ mutant; (E and F) *bclS*-complemented mutant. The filled and empty arrowheads denote the interspace filled with and lacking filamentous structures, respectively. All the images are to the same scale.

FIG 3 Fluorescence microscopic observation of BclS-GFP/mCherry fusions during sporulation. Samples were taken at different time points and analyzed by epifluorescence microscopy at a $\times 1,000$ magnification (A to O) or visualized by 3D superresolution microscopy (Q and R). (A and B) T_{-7} , with cells taken at exponential phase. (C and D) T_3 , with appearance of fluorescence in the mother cell cytoplasm. (E, F, M, and N) T_5 , where fusion proteins encasing the spore occurred on the mother cell-distal (MCD) pole of the forespore. (G and H) T_6 , where fluorescence completely engulfed the whole forespore. (I and J) T_8 , where GFP fluorescence at the mother cell-proximal (MCP) pole increased. (K, L, O, and P) T_{40} , where fluorescence encased the mature spores and was concentrated mainly at the spore core-distal (SCD) pole of the exosporium and interspace. The arrow points to the SCD pole of the exosporium and interspace. (Q and R) Superresolution 3D fluorescence micrograph and bright-field image of a BSpBUSG spore at T_{40} . Phase-contrast images are to the same scale.

mother cell-proximal (MCP) pole increased (Fig. 3I and J). The subsequent release of free spores was slow, and at T_{40} , GFP fluorescence was distributed symmetrically in the mature spores, with more fusions at the spore core-distal (SCD) pole of the exosporium and interspace (Fig. 3K and L). Cells expressing GFP only (BSpBUG) exhibited cytoplasmic fluorescence, and no obvious fluorescence was observed for the mature spores (data not shown). These results suggested that the localization of fluorescence could be due to the specific binding of BclS.

Additionally, the BclS-mCherry fusion, expressed in the recombinant BSpBUSM strain (Table 1), displayed an expression pattern and fluorescence distribution profile similar to those of the GFP fusion. Consistently, integration of BclS-mCherry started from the MCD pole of the forespore (Fig. 3M and N) and then encircled the spores, with larger amounts at the SCD pole of the exosporium and interspace (Fig. 3O and P), consistent with more electron-dense deposits being visible at the corresponding position (Fig. 2A). Further observation demonstrated that expression of BclS-GFP in the $\Delta bclS$ strain had a negative effect on the exo-

sporium, resulting in a shortened exosporium in $>99\%$ of the spores, while $>85\%$ of the mature spores expressing the BclS-mCherry fusion retained an intact exosporium (data not shown). However, little is known about the mechanisms that contribute to the morphology differences of the GFP and mCherry fusions. 3D superresolution fluorescence microscopy demonstrated a more clear localization of BclS, and the fusions were found to be located in the interspace of the spore as well as on the basal layer of the exosporium (Fig. 3Q and R). Taken together, our results suggested that BclS is required for the formation of filamentous structures, which may stretch into the interspace from the exosporium basal layer.

Spores expressing BclS-GFP or BclS-mCherry displayed fluorescence at the MCD pole of the forespore (Fig. 3E and M), from which assembly began and surrounded the spores. The localization of BclS was consistent with exosporium development in *L. sphaericus* (28) but quite different from that observed for the BclA protein, whose assembly begins from the MCP pole of the forespore (15).

Heat resistance, germination, and outgrowth of wild-type and *bclS* mutant spores. Next, we examined the roles of BclS in

FIG 4 Heat resistance of dormant spores. Spores of the wild type or the $\Delta bclS$ or *bclS*-complemented mutant were incubated at the indicated temperatures for 20 min, and the survivors were enumerated. The graph shows the means and standard deviations (SD) of results from three independent experiments. **, $P < 0.005$; ***, $P < 0.001$ (measured by Student's *t* test). RT, room temperature.

heat resistance, germination rate, and outgrowth of spores of *L. sphaericus* C3-41. For the heat resistance assay, wild-type, $\Delta bclS$ mutant, and *bclS*-complemented mutant spores were each treated at 65°C, 70°C, 75°C, or 80°C, and the survival rate was calculated by determining the plating efficiency compared to the room-temperature (RT) control. The results demonstrated that all the spores showed similar heat resistances at 65°C (Fig. 4). However, the survival rate of $\Delta bclS$ spores decreased significantly faster than did that of wild-type spores with increasing temperatures, and notably, the survival rate of $\Delta bclS$ spores at 80°C was about 3.8-fold lower than that of wild-type spores. In a similar assay, the heat resistance of spores of the *bclS*-complemented strain was partly (75°C) or totally (70°C and 80°C) restored compared to that of the $\Delta bclS$ spores (Fig. 4). These results suggested that BclS is involved in the maintenance of moist-heat resistance of *L. sphaericus* C3-41 spores.

For the germination assay, spores of the wild type, the $\Delta bclS$ mutant, and the *bclS*-complemented mutant were incubated in MBS medium for 60 min, and germination was monitored by changes in the OD₆₀₀ in combination with phase-contrast microscopic observation. As shown in Fig. 5, ~93% of wild-type spores showed a nearly complete transition from phase-bright spores to phase-dark germinated cells, with a ~38% decrease in the OD₆₀₀, compared to 49% for $\Delta bclS$ spores, which showed only a 17% decrease in the OD₆₀₀. Complementation of the *bclS* mutation resulted in a germination rate significantly higher than that for $\Delta bclS$ spores (78% versus 49%) but somewhat lower than that for wild-type spores (Fig. 5). These data indicated that BclS is required for the effective germination of spores.

It was shown previously that Bin-producing *L. sphaericus* strains were able to germinate in mosquitoes and that the final *L. sphaericus* cell counts in larval cadavers were ~1 to 1,000 times the number of spores originally ingested (21), and this recycling in larvae contributes to the persistence of mosquito biocontrol in the field. Based on our results, it is likely that BclS plays a role in the determination of persistence of spores in the environment.

The outgrowth capacities of the wild-type and $\Delta bclS$ spores were then examined, and the results showed somewhat delayed outgrowth for the mutant spores, which could be attributable to the reduced germination rate. Wild-type spores required about 2 h to resume robust growth, whereas $\Delta bclS$ spores required an additional 20 min, and the similar slopes of both curves may indicate

FIG 5 Germination analysis of spores of various *L. sphaericus* strains. (A) The decrease of the OD₆₀₀ was measured as an indicator of spore germination. (B) The percentage of germinated spores was examined by phase-contrast microscopy.

parallel vegetative growth during the next 60 min (Fig. 6). A second decrease in the OD₆₀₀ was observed for both spores, most likely as a result of the formation of visible cell aggregates, and the growth curve of the mutant culture eventually reached the level of the wild-type culture.

Conclusion. In this study, we identified and characterized a collagen-like exosporium protein of *L. sphaericus*, BclS, which was demonstrated to be essential for the formation of the filamentous structures that spread in the interspace. In contrast to other Bcl proteins of *B. cereus* group strains, such as BclA and BclB, BclS exhibited unique localization and functions. This difference may arise from both variations in the CLR length and sequence as well as divergent evolution suitable for different spore architectures.

The hydrophobic internal filamentous structures of *L. sphaericus* spores may play a role in supporting the extended and balloon-like exosporium for the enclosure of Bin toxins. Previous studies

FIG 6 Effect of the *bclS* mutation on spore outgrowth and vegetative growth.

have shown that the surface hydrophobicity of spores depends mainly on the components of the exosporium (33), and the hair-like nap had a marked effect on the hydrophobicity of *B. anthracis* spores (34). However, the filamentous structures of the exosporium may have little effect on the *L. sphaericus* spore surface, and the hydrophobicities of both the wild-type and $\Delta bclS$ spores were <10%, as determined by adherence to hexadecane (data not shown). *L. sphaericus* spores with low water hydrophobicity therefore show less tendency than *B. thuringiensis* spores to stick to and sediment with other particulates in the water column and, thus, increase their persistence in larval feeding zones (35). Nevertheless, more studies are needed to further elucidate the specific roles of BclS and the exosporium in stress tolerance and persistence of spores.

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