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Two groups of S-layer proteins, SLP1s and SLP2s, in *Bacillus thuringiensis* co-exist in the S-layer and in parasporal inclusions

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We screened four *B. thuringiensis* strains whose parasporal inclusions contained the S-layer protein (SLP), and cloned two *slp* genes from each strain. Phylogenetic analysis indicated these SLPs could be divided into two groups, SLP1s and SLP2s. To confirm whether SLPs were present in the S-layer or as a parasporal inclusion, strains CTC and BMB1152 were chosen for further study. Western blots with isolated S-layer proteins from strains CTC and BMB1152 in the vegetative phase showed that SLP1s and SLP2s were constituents of the S-layer. Immunofluorescence utilizing spore-inclusion mixtures of strains CTC and BMB1152 in the sporulation phase showed that SLP1s and SLP2s were also constituents of parasporal inclusions. When heterogeneously expressed in the crystal negative strain BMB171, four SLPs from strains CTC and BMB1152 could also form parasporal inclusions. This temporal and spatial expression is not an occasional phenomenon but ubiquitous in *B. thuringiensis* strains. [BMB reports 2011; 44(5): 323-328]

INTRODUCTION

Most Eubacteria and Archaea possess monomolecular crystalline arrays of proteinaceous subunits, termed surface layers (S-layers) (1, 2). The S-layers are formed by an entropy-driven self-assembly event in cell envelopes, and these assemble into lattices with oblique, square, or hexagonal symmetry (3, 4). The prime function of the S-layer is likely protection from viral, parasitic bacteria and enzymatic attack. Some S-layers have further evolved to be virulence determinants or attachment factors for enzymes that are useful to the cell, while others may attract scarce ions such as iron (5, 6).

S-layers are composed of a single, homogeneous protein or glycoprotein species with molecular weights ranging from 40-200 kDa. These proteins are known as S-layer proteins (SLPs) (7). Many SLPs are produced with a secretion signal peptide and an S-layer homologous (SLH) motif at the N-terminus, which is involved in anchoring the proteins to the cell surface and binding with pyruvylated secondary cell wall polymers (8, 9). The middle and C-terminal portions comprise domains that are involved in the self-assembly process (10). It is widely known that the SLP of *Lactobacillus acidophilus*, NCFM, can regulate immature dendritic and T cell functions (11). In this species, muramidase activity with antibacterial properties has been described in the S-Layer (12). In *Bacillus anthracis*, SLPs of SAP and EA1 were also reported to exhibit murein hydrolase activity (13). The SLP, SwmA, in *Synechococcus sp.* strain WH8102 is required for non-flagellar motility (14). SLH motifs could be used as carriers for the presentation of a toxin component in a live vaccine (15). Previously, we also used SLP SLH motifs as carriers to display many functional proteins on the cell surface, such as polyhistidine peptides (16), N-acyl-homoserine lactonase (17), *Mycoplasma gallisepticum* agglutinin (18), and the nucleoprotein of avian influenza virus (19).

Bacillus thuringiensis is an aerobic, spore-forming bacterium that produces crystal insecticidal proteins, which demonstrate insecticidal activities during sporulation (20). *B. thuringiensis* strain CTC has a typical S-layer, and we first isolated the *B. thuringiensis* SLP gene, *ctc* (GenBank AJ012290), from this strain (21). Another SLP gene, *ctc2* (GenBank AY460125), is adjacent and downstream of *ctc* (18, 19). Strain CTC also produced parasporal inclusions consisting of proteins that share a similar amino acid sequence to SLP, with these CTC-like strains widely distributed (22). When the *ctc* gene is expressed in acrystalliferous *B. thuringiensis* strain BMB171, it can also form parasporal inclusions (23).

There were two SLPs (CTCSLP1 and CTCSLP2) in strain CTC, and Mignot *et al.* (24) reported that *Bacillus cereus* and *B. thuringiensis* may harbor two SLP genes. Considering the constituents of the S-layer and parasporal inclusions were related with SLP, we believed it would be interesting to determine if either or both SLPs existed in the S-layer or parasporal inclusions simultaneously. In this study, we screened four *B. thuringiensis* strains whose parasporal inclusions con-

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sist of SLP (PICS strain), cloned two SLP genes from each strain and detected the existence of the two SLPs in their S-layers and parasporal inclusions.

RESULTS

Screening of *B. thuringiensis* strains whose parasporal inclusions consist of SLP

In total, 21 *B. thuringiensis* strains were cultivated during the sporulation phase and the spore-inclusion mixtures were collected for SDS-polyacrylamide gel electrophoresis (PAGE) detection. It was found that there were four strains (B22, I13, H67 and BMB1152) that were able to produce dominant 100 kDa proteins (Fig. 1A) and their N-terminal sequences showed 80-100% amino acid sequence identity with *B. anthracis* SLPs, SAP (25) and EA1 (15) (Table S2). Western blots further demonstrated that the 100 kDa proteins of these four strains could all interact with the anti-CTCSLP2-C antibody (Fig. 1A). It was confirmed that *B. thuringiensis* strain B22, I13, H67 and BMB1152 were PICS strains.

SLPs are localized to two branches of a phylogenetic tree

According to methods previously described, the *slp* genes of strain B22, I13, H67 and BMB1152 were cloned and sequenced. The nucleotide sequences of these *slp* genes (*B22slp1, B22slp2, I13slp1, I13slp2, H67slp1, H67slp2, 1152slp1, 1152slp2*) have been deposited into GenBank. All PICS strains contained two clustered *slp* genes, corresponding with the organization of the locus for *slp* genes throughout the *B. cereus* group (24). The evolutionary relationship of SLPs containing SAP and EA1 showed that these SLPs were localized to two branches of the constructed phylogenetic tree and were classified as either SLP1s or SLP2s (Fig. 1B).

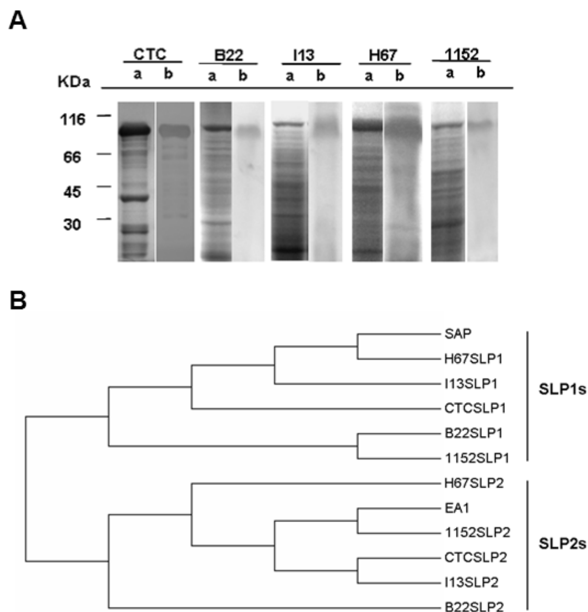

Fig. 1. (A) SDS-PAGE and Western blot analysis of SLPs. Lane a, SDS-PAGE of spore-inclusion mixtures from strain CTC, B22, I13, H67 and BMB1152; lane b, SLPs were detected by Western blot using an anti-CTCSLP2-C polyclonal antibody. (B) Phylogenetic tree of amino acid sequences for SLPs. SLPs tended to cluster among two branches (SLP1s and SLP2s). SAP and EA1 were located in branches corresponding to SLP1 and SLP2, respectively.

Primary and secondary structure prediction of SLPs showed that both groups of SLPs have three conservative SLH motifs at the N-terminus, with a signal peptide consisting of 29 amino acids. There are several distinct differences between SLP1s and SLP2s. The molecular weight of SLP1s (86-88 kDa) is smaller than that of SLP2s (91-94 kDa); the pI of SLP1s is 5-8, while for SLP2s it is 5-6; only SLP2s have a conservative anchor site, the LPXTG motif (26) towards the C-terminus along with the N-terminus SLH motif; and in SLP2s, there is 8-10% more β-sheet structure than in SLP1s.

SLP1s and SLP2s are constituents of the S-layer in PICS strains

The specificity of anti-CTCSLP1-C and anti-CTCSLP2-C antibodies was inspected. Cross-reaction of anti-CTCSLP1-C and anti-CTCSLP2-C was excluded because western blots showed that proteins produced by EMB0440 (containing p0440, harboring *ctc*-C) only hybridized to serum anti-CTCSLP1-C while proteins from EMB0441 (containing p0441, harboring *ctc2*-C) (22) only hybridized with serum anti-CTCSLP2-C (Fig. 2A).

To determine if the SLP group formed the S-layer, isolated SLPs from vegetative cells of strains CTC and BMB1152 were used as samples for western blot analysis. Both SLP1s from strains CTC and BMB1152 were expressed at high levels at 4 h post-inoculation, while SLP2s expression was much lower (Fig. 2). In strain CTC, two SLPs were steadily expressed from 8-16 h post-inoculation (Fig. 2B). In BMB1152, SLP1 expression was gradually overtaken by SLP2 expression from 8-16 h post-inoculation (Fig. 2C).

We further inspected the cell surface of strains CTC and BMB1152 at different developmental phases (8 h and 16 h). It was observed that in strain CTC, SLP1 and SLP2 were detected as a bright homogeneous layer during the exponential and stationary phases (Fig. 2D). In strain BMB1152, SLP1 was detected as a bright homogeneous layer, and SLP2 was revealed to have a punctate cell surface distribution during the exponential phase. However, the opposite was observed for SLP2, with a bright homogeneous layer observed during the stationary phase (Fig. 2E). Strain BMB171, which has no S-layer, was used as a control (Fig. 2). S-layer staining in strains B22, I13 and H67 exhibited similar patterns with that of strain CTC (results not shown).

SLP1s and SLP2s are constituents of parasporal inclusions in PICS strains

Spore-inclusion mixtures of strains CTC and BMB1152 were labeled with primary antibodies against CTCSLP1-C and

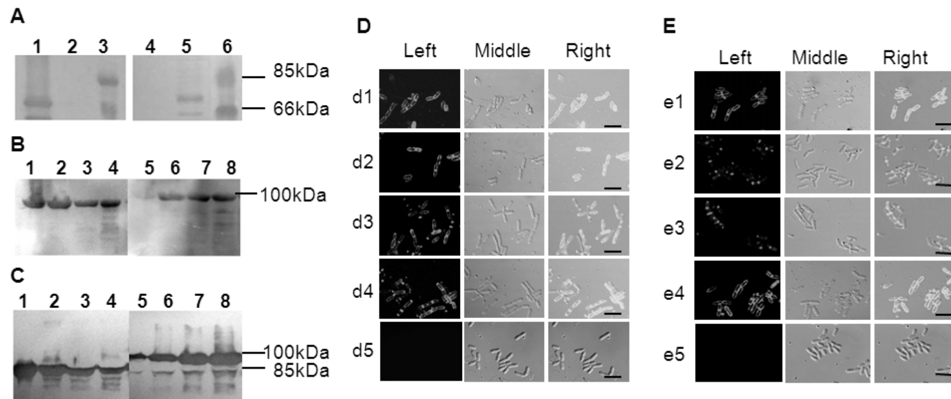

Fig. 2. (A) Western blot analysis regarding the cross-reaction between anti-CTCSLP1-C and anti-CTCSLP2-C antibodies. Lanes 1 and 4, EMB0440 crude extraction protein sample incubated with anti-CTCSLP1-C and anti-CTCSLP2-C, respectively; lanes 2 and 5, EMB0441 crude protein extraction incubated with anti-CTCSLP1-C and anti-CTCSLP2-C, respectively; lanes 3 and 6, prestained protein marker, P7708 (BioLabs). (B and C) Analysis of SLP expression at 4, 8, 12 and 16 h of growth of strain BMB1152 (B) and CTC (C) as determined by Western blot using anti-CTCSLP1-C (lanes 1-4) and anti-CTCSLP2-C (lanes 5-8). (D and E) Laser scanning confocal microscopy of *B. thuringiensis* strain CTC (D) and BMB1152 (E) at 8 h and 16 h. (D) (d1 and d2) Surface layer of strain CTC at 8 h incubated with anti-CTCSLP1-C and anti-CTCSLP2-C; (d3 and d4) surface layer of strain CTC at 16 h incubated with anti-CTCSLP1-C and anti-CTCSLP2-C; (E) (e1 and e2) surface layer of strain BMB1152 at 8 h incubated with anti-CTCSLP1-C and anti-CTCSLP2-C; (e3 and e4) surface layer of strain BMB1152 at 16 h incubated with anti-CTCSLP1-C and anti-SLP2-C; (d5 and e5) negative control BMB171 incubated with anti-CTCSLP1-C and anti-CTCSLP2-C simultaneously. Left: fluorescently stained cells, Middle: native cells, Right: overlap of fluorescence stained and native cells. Bar = 5 μm.

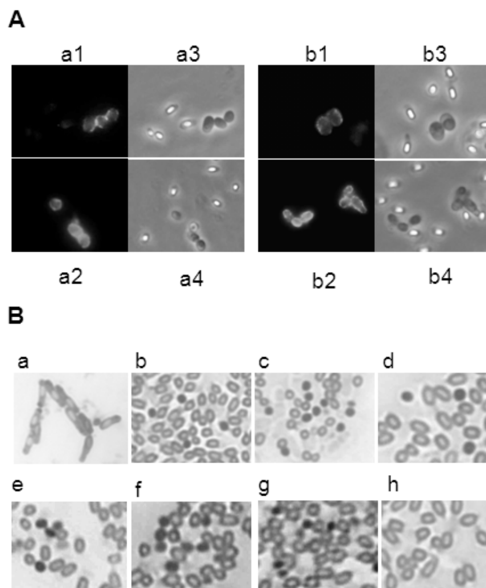

Fig. 3. (A) Photomicrograph of parasporal inclusions from *Bacillus thuringiensis* strain CTC (a1-a4) and BMB1152 (b1-b4). (a1) Parasporal inclusions of CTC hybridized with anti-CTCSLP1-C; (a2) parasporal inclusions of CTC hybridized with anti-CTCSLP2-C; (a3 and a4) light microscope images. (b1) Parasporal inclusions of strain BMB1152 incubated with anti-CTCSLP1-C; (b2) parasporal inclusions of strain BMB1152 hybridized with anti-CTCSLP2-C; (b3 and b4) light microscope images. (B) Microscopic observations of the recombinant strains. (a) YBT-020; (b) BMB0757 harboring the *1152slp1* gene; (c) BMB0758 harboring the *1152slp2* gene; (d) BMB1152; (e) CTC; (f) BMB0765 harboring the *ctc* gene; (g) BMB0766 harboring the *ctc2* gene; (h) BMB171 control.

CTCSLP2-C, followed by appropriate secondary antibodies and then visualized with a fluorescence microscope (Fig. 3A). Parasporal inclusions from strains CTC and BMB1152 exhibited a strong green fluorescent signal with both antibodies. Some spores did not exhibit a fluorescent signal, suggesting that SLPs were not present in spores of strains CTC and BMB1152 (Fig. 3A). Parasporal inclusions in strains B22, I13 and H67 had similar characteristics to strain CTC (data not shown).

Heterogeneous expression of two groups of SLPs can form parasporal inclusions

To confirm the above results, four *slp* genes (*ctc, ctc2, 1152slp1,* and *1152slp2*) were inserted into the shuttle vector pHT304, then transformed into BMB171 to yield four recombinant strains designated BMB0765, BMB0766, BMB0757, and BMB0758. These strains were cultured into the sporulation phase to inspect for parasporal inclusions. Spore-inclusion mixtures were observed *via* light microscopy. The results showed that all four recombinant strains could form parasporal inclusions (Fig. 3B). Strain BMB171 was used as a control, exhibiting no parasporal inclusions.

DISCUSSION

Until now, only a few *slp* genes from *B. thuringiensis* have been cloned. In this study, we obtained eight new *slp* genes from *B. thuringiensis* PICS strains B22, I13, H67 and BMB 1152. The constructed phylogenetic tree based upon the ami-

Table 1. Strains and plasmids used in this study

Strains or plasmids	Characteristics	Origin or reference
Strains		
CTC	*B. thuringiensis* subsp. *finitimus*, serotype H2	(21)
B22	*B. thuringiensis*, serotype unknown	Stored in our lab
I13	*B. thuringiensis*, serotype unknown	Stored in our lab
H67	*B. thuringiensis*, serotype H67	Stored in our lab
YBT-020	*B. thuringiensis* subsp. *finitimus*, serotype H2	(22)
BMB1152	Plasmid curing mutant of strain YBT-020	(28)
BMB171	Acrystalliferous *B. thuringiensis* mutant	(29)
DH5α	*E. coli, supE44 lacU169 (80lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Stored in our lab
BL21 (DE3)	*E. coli, hsd*S gal (λcIts857 *ind*1 *Sam7 nin*5 "Origami")	Stored in our lab
BMB0765	Recombinant strain BMB171, haboring pBMB0765	This study
BMB0766	Recombinant strain BMB171, haboring pBMB0766	This study
BMB0757	Recombinant strain BMB171, haboring pBMB0757	This study
BMB0758	Recombinant strain BMB171, haboring pBMB0758	This study
Plasmids		
pHT304	*E. coli* and *B. thuringiensis* shuttle vector; Amp^r, Erm^r	(29)
pET-28a	Expression vector; Kar^r	Stored in our lab
pEMB0440	C terminal half of *ctc* gene cloned into pET-28a; Kar^r	This study
pEMB0441	C terminal half of *ctc2* gene cloned into pET-28a; Kar^r	(22)
pBMB0765	*ctc* gene cloned into pHT304; Amp^r, Erm^r	This study
pBMB0766	*ctc2* gene cloned into pHT304; Amp^r, Erm^r	This study
pBMB0757	*1152slp1* gene cloned into pHT304; Amp^r, Erm^r	This study
pBMB0758	*1152slp2* gene cloned into pHT304; Amp^r, Erm^r	This study

no acids for these SLPs showed that all SLPs located on the two branches were designated as SLP1s and SLP2s (Fig. 1B). Mignot *et al.* (24) showed that in all *B. cereus* strains harboring S-layers, their *slp* genes were either homologs to *eag* or to *sap* and *slpA*. In our classification scheme, the *eag* gene was classified as *slp2s,* and *sap* as *slp1s*. Primary and secondary structure prediction of these two groups of SLPs showed some typical differences between SLP1s and SLP2s. The functions of the two SLPs may be different but are similar in terms of forming the S-layer and parasporal inclusions.

Typical parasporal inclusions of *B. thuringiensis* are usually encoded by *cry* genes and function as insecticidal toxins. The function of parasporal inclusions encoded by the *slp* gene is unclear. Only the SLP in strain GP1 was reported to have any pesticidal activity against *Epilachna varivestis* (27). Comparative sequence analysis of SLP and insecticidal crystal protein (Cry protein) demonstrated no homologies (data not shown). It is possible these two kinds of parasporal inclusions have similar pesticidal activity but are different in their insecticidal mechanism. When the *slp1* and *slp2* genes were heterogeneously expressed in strain BMB171, they were each able to form parasporal inclusions. We inspected the cell surfaces of recombinant strains harboring *slp1* and *slp2* genes, but no homogeneous S-layer was found (data not shown). It is possible that formation of parasporal inclusions was a simpler process for the cell to undertake as compared to the S-layer, which may require more elements. It has been reported that a two gene operon, *csaAB*, is needed for cell surface anchoring as the CsaB protein is involved in the addition of a pyruvyl group to a peptidoglycan-associated polysaccharide fraction, which is necessary for surface binding of the SLH domain (8). It may be necessary to have both the *csaAB* operon and the SLP gene to form the S-layer and parasporal inclusions simultaneously.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *B. thuringiensis* strains CTC, B22, I13 and H67 were isolated from soil samples collected by our laboratory.

B. thuringiensis stains were grown in brain heart infusion medium (Difco Laboratories) or 2YT medium (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl, 1.5% agar, pH 7.2-7.4) at 28°C. *Escherichia coli* DH5α and BL21 (DE3) were grown in Luria-Bertani (LB) medium at 37°C and were used as hosts for plasmid construction and gene expression. Antibiotic concentrations for bacterial selection were 100 μg ml^{-1} for ampicillin, 25 μg ml^{-1} for erythromycin and 25 μg ml^{-1} for kanamycin (30).

Antibody preparation

An antibody against CTCSLP2-C, the C- terminal half of strain CTC SLP2 protein, was described as anti-CTCSLP-C previously (22). The antibody against CTCSLP1-C, was produced in a manner similar to anti-CTCSLP2-C, with a amplification of 1.8 kb portion of the *ctc* genes encoding the C-terminal half of CTCSLP1 protein from the genomic DNA of strain CTC using primers P23 and P24 (Table S1).

Chemical extraction of S-layer protein

B. thuringiensis strain CTC was grown in brain heart infusion

broth medium (Difco) and the cell surface-anchored proteins were extracted as described by Pena *et al.* (27). The samples were then made western blot detection using anti-CTCSLP1-C and anti-CTCSLP2-C polyclonal antibodies as described above.

N-terminal protein sequencing
N-terminal sequencing of the parasporal inclusion proteins was performed after separation on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) in a semidry transfer chamber following the manufacturer's instructions, and then applied to an ABI 491 Protein Sequencer (Applied Biosystems).

Cloning and sequencing of SLP coding genes
Based on the conserved sequences of *slp* genes and their flanking sequences of *B. cereus* group, primers were designed. The sequence of primers used in this study and the amplification strategy are shown in Table S1. Following amplification, the PCR products were purified and sequenced (AuGct Company).

Phylogenetic tree and structure predictions
Amino acid sequence alignments and phylogenetic trees for SLPs were produced by the MEGA 4.1 software (http://www.megasoftware.net/). Analysis of primary and secondary protein structure predictions was made by the PHD software (http://www.predictprotein.org/).

Immunofluorescence labeling
To inspect the S-layer, 100 μl liquid cultures were collected at 8 h and 16 h. To inspect the parasporal inclusions, strains were cultivated on 2YT solid-phase medium into the sporulation phase and then 10 mm^3 cultures were collected. All samples were transferred into 1.5 ml microcentrifuge tubes and washed three times using phosphate-buffered saline (PBS). Immunofluorescence labeling was conducted according to the methods described by McCarren and Brahamsha (14) with some modifications. After blocking overnight at 4°C with PBS and 3% BSA (Sigma), samples were incubated for 1 h at 37°C with primary antibodies diluted 1 : 500 in blocking solution, washed five times with PBS and once with blocking solution and then incubated with FITC-labeled goat anti-rabbit IgG diluted 1 : 50 in blocking solution for 1 h at 37°C. Following another three washes with PBS, samples were equilibrated for 5 min in glycerin buffer (glycerin : PBS, 9 : 1) for fluorescence microscope observation.

Microscopic observation
For light and fluorescence microscopy, observation was performed with an Olympus Photomicroscope. S-layer morphology observation was observed using a Zeiss laser scanning confocal microscope (LSCM510 META, Zeiss).

S-layer proteins expressed in BMB171
Four *slp* genes from strain CTC and BMB1152 were amplified by PCR (Table S1). The amplicons were inserted into the corresponding *Kpn*I/*Sph*I or *Eco*RI/*Sph*I site of the shuttle vector pHT304 (28). Then p0765 (pHT304 harboring *ctc*), p0766 (pHT304 harboring *ctc2*), p0757 (pHT304 harboring *1152 slp1*), and p0758 (pHT304 harboring *1152slp2*) were obtained and transformed into *B. thuringiensis* strain BMB171 by electroporation (29).

Nucleotide sequence accession numbers
The nucleotide sequences of the S-layer protein genes of *ctc, ctc2, B22slp1, I13slp1, H67slp1, 1152slp1, B22slp2, I13slp2, H67slp2, 1152slp2,* have been deposited in the GenBank database under the accession numbers of AJ012290, AY460125, GU266553, GU266554, GU266555, GU266556, GU266557, GU266558, GU266559 and GU266560, respectively.

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