Production and cell surface display of recombinant anthrax protective antigen on the surface layer of attenuated Bacillus anthracis

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ORIGINAL PAPER

Production and cell surface display of recombinant anthrax protective antigen on the surface layer of attenuated *Bacillus anthracis*

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Abstract To investigate the surface display of the anthrax protective antigen (PA) on attenuated *Bacillus anthracis*, a recombinant *B. anthracis* strain, named AP429 was constructed by integrating into the chromosome a translational fusion harboring the DNA fragments encoding the cell wall-targeting domain of the S-layer protein EA1 and the anthrax PA. Crerecombinase action at the loxP sites excised the antibiotic marker. Western blot analysis, fluorescence-activated cell sorting and immunofluorescence analysis confirmed that PA was successfully expressed on the S-layer of the recombinant antibiotic marker-free strain. Notwithstanding extensive proteolytic degradation of the hybrid protein SLHs-PA, quantitative ELISA revealed that approximately 8.1×10^6 molecules of SLHs-PA were gained from each *Bacillus* cell. Moreover, electron microscopy assay indicated that the typical S-layer structures could be clearly observed from the recombinant strain micrographs.

Keywords *Bacillus anthracis* · Cre-*loxP* system · Protective antigen · S-layer · Surface display

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Introduction

The surface-layer (S-layer) protein is a type of bacterial surface protein anchored on the outmost cell surface of bacteria (Sleytr and Beveridge 1999). The interaction between the cell wall and the S-layer protein is very strong, although not covalent. Thus, the high level of expression of this protein, together with its efficient binding to the cell wall, renders it ideal for studying cell surface anchoring and developing new expression systems (Sleytr et al. 2007). Due to the ability to express heterologous proteins on the cell surface, some bacteria with S-layer have become an essential component of industrial catalysts, sorbents, sensors, vaccine delivery vehicles, and screening platforms (Messner et al. 2008; Ilk et al. 2011; Hollmann et al. 2010; Schneewind and Missiakas 2012). Based on published data, researchers are giving this technology greater attention.

Bacillus anthracis, a Gram-positive spore-forming bacterium, is the causative agent of anthrax (Mock and Fouet 2001). The protective efficacies of existing anthrax vaccines vary significantly against different *B. anthracis* isolates in several animal models and are not very satisfactory (Fellows et al. 2001; Mock and Fouet 2001; Chitlaru et al. 2011). Although the licensed human anthrax vaccines AVA and AVP are effective, their undefined composition, lot-to-lot variation, and extensive dosing regimens have served as the impetus for the development of second-generation vaccines, including live attenuated ones (Feinen et al. 2014; Kaur et al. 2013; Cote et al. 2012; Bouzianas 2010; Mohamadzadeh et al. 2009; Osorio et al. 2009; Tournier et al. 2009; Friedlander and Little 2009). Allowing for the availability of an S-layer in *B. anthracis*, the surface display of antigens has been widely considered. *B. anthracis* simultaneously synthesizes two S-layer proteins, namely, extracellular antigen 1 (EA1) and surface array protein (Sap). For previous studies have shown that Sap

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Table 1 Plasmids and strains used in this study

Plasmids and strains	Relevant characteristics	Source
Plasmids		
pT-loxP::spc	A spc^r with two directly repeated loxP sites of each end inserted the T-vector pEASY-T1	(Wang et al. 2011)
pT-SLH-PA::spc	T-Vector with fragment of slhs-pag-loxP-spc-loxP	This work
pMAD	Shuttle vector, Amp^r in *E. coli* and Em^r in *B. anthracis*, Ts	(Arnaud et al. 2004)
pMAD-SLHs-PA::spc	pMAD with fragment of SLH-PAg-loxP-spc-loxP	This work
pHY-cre	Expression vector of Cre recombinase	(Wang et al. 2011)
B. anthracis strains		
A16R	pXO1$^+$ pXO2$^-$, China vaccine strain.	This lab
AP422	pXO1$^-$pXO2$^-$, deriving from A16R, China vaccine strain.	This lab, CGMCC No. 1569
AP429	AP422::PA, AP422 with partial *eag* gene replaced by the sequence encoding the PA20 fragment.	This work
E. coli strains		
Top10	*F-lacX74 recA1 araD139Δ (mcrA Δ(mrr-hsd RMS-mcrBC) φ80 lacZΔ M15Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG*	TianGen
SCS110	*rpsL* (Str^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) (F́ *traD36 proAB lacI^q ZΔM15*)	Stratagene

is the major protein present in culture supernatants, whereas EA1 is exclusively associated with cells at the stationary phase (Mignot et al. 2002; Mesnage et al. 1997), EA1 has been regarded as a novel anchoring protein (Mesnage et al. 1999). In addition, using this system, surface display antigens are better recognized by the host immune system, thus creating better immune responses, and elicit strong, long-lasting immunity; in addition, the components of the host may contribute to a very strong immune response acting as an adjuvant to recombinant antigen proteins (Nhan et al. 2011).

Using a nontoxinogenic and nonencapsulated *B. anthracis* platform, a previous study tested the 20-kDa N-terminal fragment of the anthrax protective antigen (PA) protein on the S-layer of attenuated *B. anthracis* and the recombinant strain was evaluated as a candidate vaccine (Wang et al. 2011). In the present study, we explored the possible expression of full-length mature PA with a molecular weight of 83 kDa, much higher than that of PA20. The PA was efficiently expressed on the S-layer of the AP422 (pXO1$^-$, XO2$^-$) strain when fused to SLH motifs of EA1 despite the occurrence of degradation. These results demonstrate that this system can be used as a display system to present S-layer proteins or the fused tags.

Materials and methods

Bacterial strains and growth conditions

All bacterial strains used in this study and their relevant characteristics are listed in Table 1. All *B. anthracis* strains are derivatives of the Chinese vaccine strain A16R. Bacteria were aerobically grown at 30, 37, or 42 °C. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth and used as hosts for plasmid cloning. *B. anthracis* strains were grown in brain heart infusion agar with 0.5 % glycerol (BHIG, BD, USA) or LB medium. Antibiotics (Merck, Germany) were added to the medium when appropriate to give the following final concentrations: 100 μg/mL ampicillin for *E. coli*; 400 and 5 μg/mL erythromycin (EM) for *E. coli* and *B. anthracis*, respectively; and 50 and 300 μg/mL spectinomycin (Spc) for *E. coli* and *B. anthracis*, respectively.

DNA manipulation

Preparation of the plasmid DNA from *E. coli*, *E. coli* transformation, and recombinant DNA techniques were performed using standard procedures. *E. coli* TOP10 competent cells were obtained from TianGen (China), whereas *E. coli* SCS110 competent cells were purchased from Stratagene (USA). Recombinant plasmid construction was conducted on *E. coli* TOP10. Chromosomal DNA of *B. anthracis* was isolated using a Wizard genomic purification kit (Promega, USA) according to the protocol for isolation of genomic DNA from Gram-positive bacteria. *B. anthracis* was electroporated with unmethylated plasmid DNA isolated from *E. coli* SCS110. Electroporation-competent *B. anthracis* cells were prepared as previously described (Shatalin and Neyfakh 2005). 5 μL of plasmid DNA (1 μg n water) was added to 100 μL aliquot of competent cells and pulsed (2.5 kV, 25 μF, 200 Ω) in a

0.2 cm gap cuvette. The cells were resuspended in 1 mL BHIG and incubated for 1.5 h at 30 °C with aeration. Recovered cells were spread on LB agar plates, containing Em.

Construction of the recombinant strain

The plasmid for homology recombinant and *B. anthracis* mutants were constructed according to the gene targeting methods used in previously published work (Wang et al. 2011). A 5.0-kb DNA fragment containing the SLH motifs coding sequence of the *B. anthracis eag* gene, *pagA* fragment (amplified by polymerase chain reaction using the *B. anthracis* A16R chromosome as template and PAF and PAR oligonucleotides—CCAATGCATGAAGTTAAACA GGAGAA and—TCCCCGCGGTTATCCTATCTCAT, respectively), spc^r fragment, and 0.8-kb downstream *eag* gene was inserted into plasmid pMAD to create pMAD-SLHs-PA::spc. The identified pMAD-SLHs-PA::spc was used for the insertion of the sequence encoding the *pagA* fragment under the control of the *eag* gene. The constructed plasmids were identified by restriction endonuclease digestion and agarose gel electrophoresis. Next, the constructed plasmid was introduced by electroporation into *B. anthracis* AP422, after which transformants were selected under strict temperature and antibiotic pressure.

For elimination of Spc resistance, plasmid pHY-cre was electroporated into the isolates with selection for EM resistance at 30 °C. EM-resistant colonies were transferred to antibiotic-free agar and incubated at 37 °C to eliminate pHY-cre. The presence of a single loxP site within the targeted gene (s) of the fully antibiotic-sensitive recombinant strain was confirmed by PCR and/or sequence analysis.

Detection of the expression of PA

Bacillus anthracis cells were grown in 400 mL of LB medium for 12 h and washed once with 20 mM Tris buffer (pH 8.0). Bacterial pellets were resuspended using 1/10 volume of Tris buffer and briefly sonicated followed by centrifugation (20,000×*g* for 20 min at 4 °C). The pellet (S-layer protein) was resuspended using 40 mL of Tris buffer. For secretion protein, the supernatant fraction was collected from the bacterial culture described above by filtering with a 0.45-μm syringe filter. The filtered growth medium was subjected to 10 % trichloroacetic acid precipitation (4 °C overnight), followed by centrifugation, after which the pellet was resuspended in 0.1 NaOH. Equivalent volumes (25 μL) of medium (concentrated), lysate, and S-layer protein fractions were subjected to 12 % SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred to an NC membrane (Amersham Biosciences, UK) using the Bio-Rad Trans-Blot system. The membrane was incubated in blocking solution containing 5 % fat-free milk for 1 h and then incubated with the mouse αPA N-terminal monoclonal antibodies (1:5,000 dilution; provided by Prof. Duan Qing, Beijing institute of microbiology and epidemiology), αPA C-terminal polyclonal antibodies (1:200 dilution; Santa Cruz, USA), and αL6 (L6, a ribosomal protein in the cytoplasm) rabbit antiserum (1:10,000 dilution; this work). After being washed, the membrane was incubated with the measurable IgG conjugated to HRP (diluted 1:5,000; Jackson, USA). The bound antibody was detected using an HRP Western blot analysis system (TianGen, China).

Immunofluorescence

Immunofluorescence analysis was mainly performed according to the protocol described previously (Wang et al. 2011). Bacteria grown on brain heart infusion agar with 0.5 % glycerol were washed with 20 mM phosphate buffered saline (PBS) (pH 7.4), applied to a micro-cover glass slide, air-dried, and fixed in 2 % formaldehyde for 10 min. The bacteria were washed twice with PBS and incubated for 1 h with mouse αPA N-terminal monoclonal antibodies (1:100, v/v). Bacteria were then washed twice with PBS-bovine serum albumin (BSA) (1 % BSA, wt/vol), and the primary antibodies were detected by incubating slides with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:4,000, v/v; Abcam, UK) for 1 h. The bacteria were washed four times with PBS-BSA and mounted on a laser scanning microscope (Zeiss LSM510 Meta; Germany).

Fluorescence-activated cell sorting (FACS)

Flow cytometry analysis was performed to confirm the surface localization of the recombinant PA fusion proteins. An overnight culture of AP422 as the negative control and the recombinant cells were harvested and washed thrice with PBS, resuspended in PBS containing 1 % skim milk and αPA N-terminal monoclonal antibodies (1:100), and incubated on ice for 1.5 h. After being washed thrice with PBS, the cells were incubated with FITC-conjugated goat anti-mouse IgG (1:1,000, v/v; Abcam) on ice for 1 h. The FITC-labeled cells were examined using a FACS can flow cytometer and FACSCalibur (BD Biosciences, USA).

Quantitation of PA

First, the AP429 strain and A16R strain (Chinese vaccine strain) were grown in an LB medium. The fractions of culture, including the supernatant, lysate, and S-layer, were separated according to the above-described protocol. Samples were subjected to SDS-PAGE and Western blot

analysis. PA expressed in different strains was compared using the gray scale as reference. At the same time, using the commercial rPA as the standard substance, an accurate quantitative detection of total fusion protein SLHs-PA was performed by sandwich ELISA (Wie et al.1982; Gan et al. 1999). *B. anthracis* AP429 strains were grown in 400 mL of LB medium for 12 h. Next, the OD_{600} was detected and the CFU was determined by plating serial dilutions on LB agar plates. After treatment with sonication, 20 mL of bacterial culture was used to obtain protein samples for the ELISA test succedent. Triplicate 100-μL aliquots of these samples diluted with carbonate/bicarbonate buffer (serial twofold dilutions) were added to a 96-well microtiter plate (Costar, USA) and incubated in a humid atmosphere for 12 h at 4 °C. The blank was 100 μL of buffer. The plate was then washed three times in an automatic washer (Thermo Wellwash, USA) with PBS containing Tween-20. The plate was blocked with 200 μL of fat-free milk (5 % in PBS with Tween-20), incubated at 37 °C for 2 h, and then washed as before. It was then incubated (37 °C for 2 h) with 100 μL of αPA N-terminal monoclonal antibodies (1:10,000 dilution) or anti-PA polyclonal antibodies (1:500 dilution). After washing, 100 μL of measurable IgG conjugated to HRP (1:5,000 dilution) was added, followed by incubation (37 °C for 1 h) and then automatic washing of the plate. After washing, 100 μL of TMB substrate solution (TianGen, China) was added to the wells in the microtiter plate. After 10 min at 37 °C, the reaction was interrupted by adding 50 μL of 1 M H_2SO_4. Absorbance was then read at 450 nm. For the calibration curve, 100-μL aliquots of PA protein, equivalent to 100, 50, 25, 12.5, 6.25, and 3.125 ng, were also executed using the above-described program. According to the results of ELISA and bacteria count, the number of SLHs-PA molecules on each bacterium (N) was calculated based on the following equation, $N = (C_{PA}/Mr) \times NA \times C_{BA}^{-1}$. [Note: NA: Avogadro' constant, 6.02×10^{23}; Mr: Relative molecular mass of PA, 8.3×10^4; C_{PA}: concentration of PA (g/mL); C_{BA}: cell concentration (CFU/mL)].

Extraction and purification of S-layer proteins

One hundred milliliters of *B. anthracis* grown overnight was centrifuged (8,000×*g* for 20 min at 4 °C) and then washed once with cold distilled water. The wet bacterial pellet was suspended in 20 mM Tris buffer (pH 8.0) supplemented with protease inhibitor cocktails (Amresco, USA) and briefly sonicated. Following three washes of the pellet with 20 mM Tris buffer (pH 8.0), the insoluble material was resuspended in 5 mL of LiCl in the same buffer and then centrifuged (20,000×*g* for 20 min at 4 °C). The supernatant was harvested after centrifugation. Dialysis of the supernatant against distilled water was conducted in a 4 °C cold room with frequent changes of the water during the first 4 h of dialysis of its total duration of 24 h. During dialysis, a white precipitate characteristic of reassociated insoluble paracrystalline S layers appeared. The precipitate was harvested by centrifugation (20,000×*g* for 20 min at 4 °C). The pellet was then resuspended and stirred in 1 mL of LiCl for 15 min at 4 °C and centrifuged at 20,000×*g* for 20 min at 4 °C. Each pellet (pure S-layer protein) was washed with cold sterile distilled water three times and resuspended in distilled water.

Electron microscopy

Bacillus anthracis cells were grown in LB medium for 12 h, harvested by centrifugation at 10,000×*g*, washed with 20 mM Tris buffer (pH 8.0), and disrupted by sonication for 10 s. The pretreated strains and purified S-layer protein were used for electron microscopy detection. Negative staining was performed on 300 copper-mesh grids with glow-discharged carbon support film. Grids were floated on a droplet of the cell-envelope suspension and then on another droplet of 2 % uranyl acetate. Excess fluid was removed with filter paper. Micrographs were recorded with HITACHI H-7650 (HITACHI, Japan) operating at 80 kV.

Results

Construction of recombinant strains

The *eag* gene, coding the S-layer protein EA1, was replaced partly with *pagA* to obtain recombinant *B. anthracis* expressing PA on the S-layer. The procedure is described in Fig. 1a and b. *pagA* was fused with the sequence of *eag* coding SLH motifs and under the control of the *eag* promoter. Positive colonies with double-crossover events were identified by PCR. A thermosensitive plasmid expressing Crerecombinase was introduced at a permissive temperature to remove the antibiotic marker, and Crerecombinase action at the loxP sites excised the Spc resistance cassette. The final derivative strain was identified by PCR as well as sequencing analysis and named AP429.

Expression and surface exposure of the hybrid protein SLHs-PA

The AP429 strain was grown in LB medium. The fractions of culture, including the supernatant, lysate, and S-layer, were separated according to the protocol shown in materials and methods section. Samples were subjected to SDS-PAGE and Western blot analysis. The SLHs-PA hybrid protein was found in all samples of recombinant strain AP429, but not in the control AP422 samples (Fig. 2).

Immunofluorescence experiments showed that the hybrid protein SLHs-PA, expressed by the AP429 strain, was immobilized onto the cell surface of the recombinant bacilli. In contrast, the control strain AP422 was observed to express less green fluorescence (Fig. 3).

FACS assay also was performed with αPA monoclonal antibodies and provided evidence for surface localization.

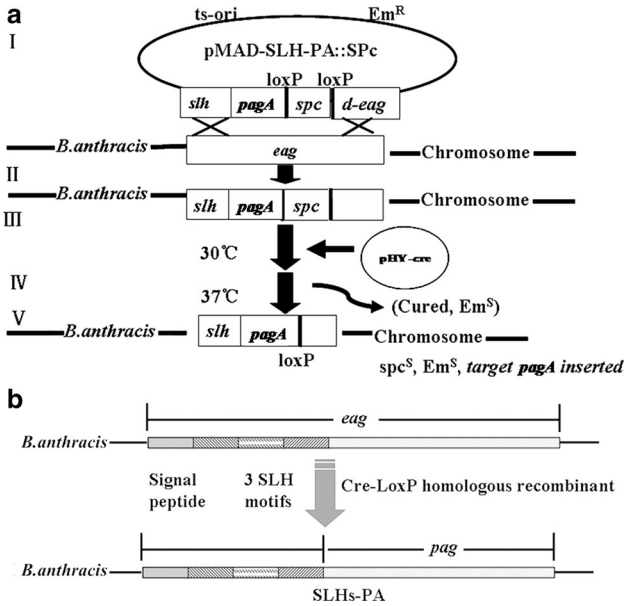

Fig. 1 Procedure for knockin of the *pagA* gene in *B. anthracis*. The complete route of the gene manipulation was shown in (**a**). (*I*) The targeted plasmid is transformed into *B. anthracis*. (*II*) The allelic exchange event is selected by the spc^r phenotype, accompanied by loss of the pMAD-SLHs-PA::spc plasmid. (*III*) Removal of the spc^r cassette from the chromosome is achieved by Cre-mediated recombination. (*IV*) Elimination the Cre recombinase-producing vector pCrePA. (*V*) The result is replacement of a portion of the *eag* gene by *pagA* and forming a new gene coding hybrid SLHs-PA. **b** The non-SLHs motif part of EA1 protein was substituted by PA protein and gained the hybrid protein SLHs-PA as described

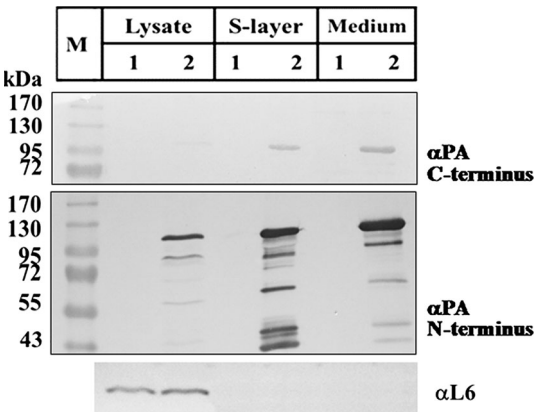

Fig. 2 Expression of PA in mutant strains AP429. Western blot analysis was executed using αPA C-terminal antibodies and αPA C-terminal antibodies as the primary antibody respectively. *Lane M*, stained protein marker; *lane 1*, AP422; *lanes 2*, AP429

As shown in Fig. 4, under flow cytometry, a distinctive and homogenous increase in fluorescence was observed in AP429 cells compared with the control strain (shift of histogram to the right, peak 2 in Fig. 4), indicating that the SLHs-PA hybrid protein is homogeneously displayed onto the surface of the recombinant bacilli.

Quantification of the SLHs-PA hybrid protein

Western blot analysis was also performed with PA N-terminal monoclonal antibodies used as primary antibodies to compare the PA protein content in AP429 with that in A16R. The results are shown in Fig. 5. Under the same conditions, PA was found in supernatant, lysate, and S-layer protein samples of AP429 and mainly in supernatant samples of A16R. The expression level of PA in AP429 was much higher than that in A16R strains under the same culture conditions.

A quantitative determination of the amount of SLHs-PA exposed on *B. anthracis* S-layer was obtained by Quantitative sandwich ELISA experiments using serial dilutions of purified PA. With the use of this optimal ELISA procedure (see Materials and methods), calibration curves with excellent reproducibility were obtained with the PA protein. The production of PA from AP429 cultured in LB medium in a shake flask was also measured using ELISA as described above. The ELISA standard curve of PA was based on six calibration standards obtained by a twofold dilution of PA standards ranging from 31.25 to 1,000 ng/mL (Fig. 6).They showed a good correlation ($R^2 = 0.995$). This range was defined as the working range of the assay. The quantitative results of ELISA suggested that approximately 190 μg of equivalent PA protein was extracted from 1 mL of culture sample when the degradation phenomenon was ignored. Based on the bacterial number (1.7×10^8 CFU/mL) and molecular weight of PA (83 kDa), we estimated that 8.1×10^6 molecules of SLHs-PA proteins (including those with a PA protein fragment) were gained from each AP429 cell. The results obtained using polyclonal antibodies were not satisfactory (data not shown), making it very difficult for us to calculate the amount of complete SLHs-PA proteins.

Functional assay

To determine the S-layer-forming function of the hybrid S-layer protein expressed on the surface of bacteria, we performed electron microscopy assay. *B. anthracis* AP429 cells were grown in an LB medium for 12 h and pretreated according to the protocol described in Materials and methods. The typical S-layer structures could be clearly observed from this strain in the electron micrographs. Thus, the SLHs-PA did not appear to affect the ability of host bacteria to form the S-layer lattice in vivo

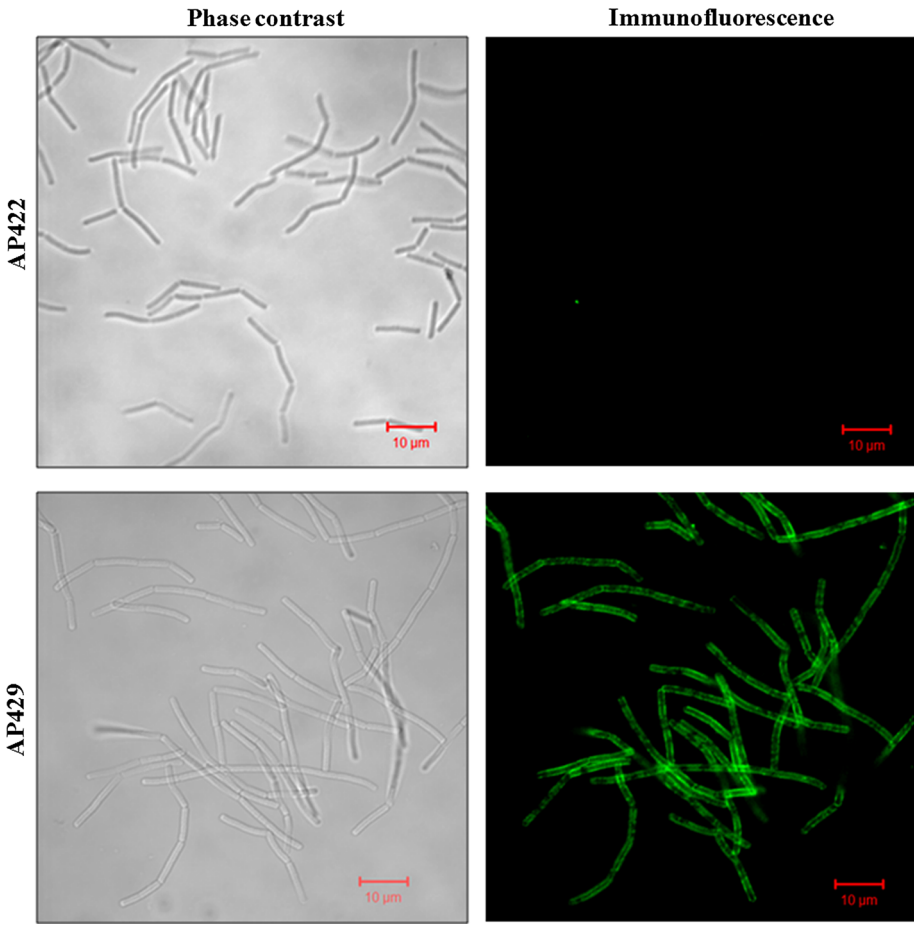

Fig. 3 Immunofluorescence microscopic analysis of whole AP422 and AP429 cells. Vegetative cells are visualized by phase contrast and by immunofluorescence microscopy. Samples were labeled with mouse αPA antibodies, followed by anti-mouse IgG-FITC conjugates. The *scale bar* represents 10 μm

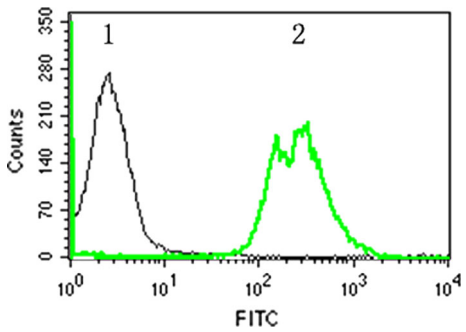

Fig. 4 FACS histograms of recombinant AP422::PA cells for determining the surface localization of the SLHs-PA hybrid protein. 1, AP422 cell (used as negative control); 2, AP429

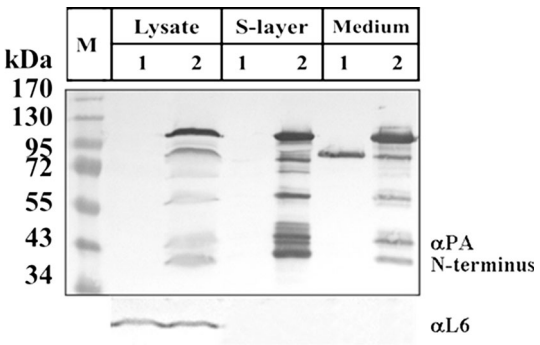

Fig. 5 Compare the expression level of PA in AP429 with in A16R strain by Western blot analysis. The test was executed using αPA20 antibodies as the primary antibody. *Lane M*, stained protein marker; *lane 1*, A16R; *lanes 2*, AP429

(Fig. 7a).However, the typical S-layer structures were not observed when the hybrid S-layer proteins were reassembled in vitro (Fig. 7b). The reasons for this phenomenon need to be further explored.

Discussion

In this study, Western blot analysis yielded distinguishing results when different antibodies were used. A series of proteins with different molecular weights were detected (many of them with a molecular weight somewhat lower than expected) when the αPA N-terminal monoclonal antibodies were used, whereas only one main protein bond was detected when the αPA C-terminal polyclonal antibodies were used. These results suggested that the hybrid protein SLHs-PA showed signs of extensive proteolysis. The instability of the hybrid protein-based S-layer EA1 had

been described in previous work (Mesnage et al. 1997), which suggested that the instability of these proteins may be an intrinsic property of the SLH motifs for proteases. However, according to our results, the PA fragment of the hybrid protein also showed severe proteolytic degradation. As the truncated proteins were detected in supernatant, lysate, and S-layer protein, we assumed that the proteolytic degradation of the hybrid protein PA possibly occurred when the protein was secreted and assembled on the bacterial S-layer, although PA is the natural protein of *B. anthracis*. Analogously, Pomerantsev et al. 2011 also revealed that secretion of mass extracellular metalloproteases, such as NprB, InhA1 and InhA2, TasA, MmpZ, and camelysin, might significantly lower the levels of intact anthrax toxin components in the *B. anthracis* secretome.

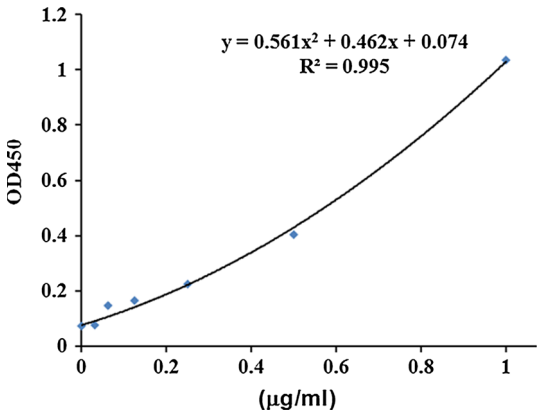

Fig. 6 The standard curve of the PA double sandwich ELISA. Twofold sequential dilution of PA in carbonate/bicarbonate buffer were evaluated in several replicate quantitations with this ELISA system. OD readings were determined at 450 nm. The graph demonstrates a representative *curve* from a typical assay

We thus hypothesized that the PA fragment of the hybrid protein would also be cleared by these proteases not only in culture medium but also on the S-layer.

Crystalline bacterial cell S-layers have been identified in a considerable number of bacterial species. S-layer lattices can serve as a matrix for the controlled immobilization of functional biomolecules; more recently, the construction of S-layer fusion proteins has been shown to combine the self-assembly principle with a broad spectrum of specific functions (e.g., ligands, antibodies, antigens, and enzymes), providing unsurpassed precision in spatial control and alignment of functions encoded in proteins(Sleytr et al. 2007). For *B. anthracis*, two abundant surface proteins, EA1 and Sap, serve as the main components of the S-layer. Previous studies have shown that EA1 is exclusively associated with cells and that the promoters of the *eag* gene efficiently express the genes under its control within the host once germination has occurred (Mesnage et al. 1997). Therefore, the S-layer homology domain of EA1 is considered a means for anchoring heterologous proteins on the cell surface of *B. anthracis*. Microbial cell surface display has many potential applications, including live vaccines. The surface display of vaccines as part of an S-layer would thus be a very efficient way of presenting antigens to the mucosa-associated lymphoreticular tissue. The surface display system developed in this study might be used to develop live attenuated *B. anthracis* veterinary vaccines.

In conclusion, PA was efficiently expressed at a high-level on the S-layer of *B. anthracis* when fused to the SLH motifs of EA1. The results indicate that the SLH motifs are appropriate for the display of heterologous proteins on the S-layer, even if the molecular weight of the target protein is high. The hybrid protein SLHs-PA can carry out the biological function of the S-layer in vivo. More importantly,

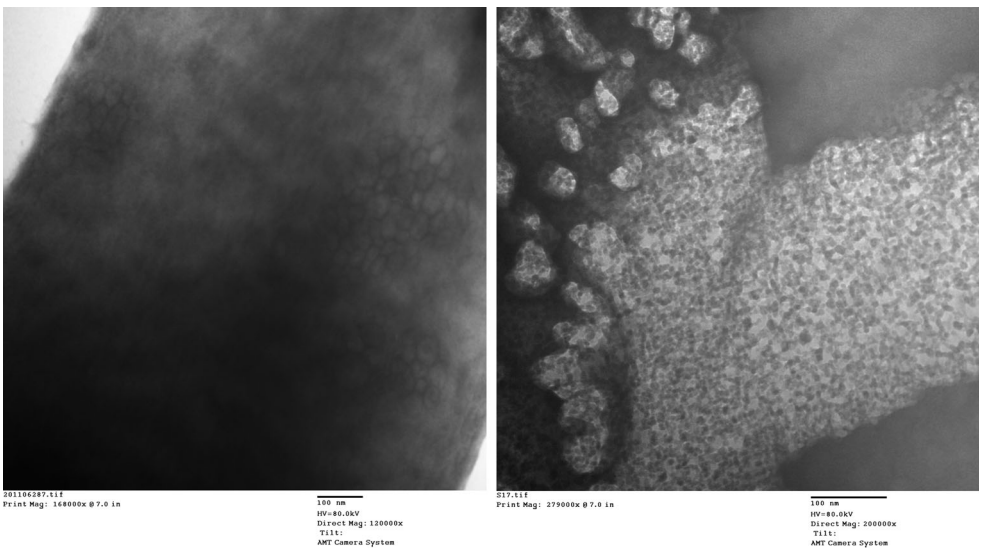

Fig. 7 Electron micrograph of AP429 strain (**a**) and the reassembled hybrid S-layer proteins (**b**). Compared with the proteins, the negatively stained AP429 preparation showed a typical S-layer structure

this antibiotic marker-free strain might be significant for future development of new live attenuated veterinary anthrax vaccines.

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Conflict of interest The authors declare that they have no competing interests.

References

Arnaud M, Chastanet A, Debarbouille M (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. Appl Environ Microbiol 70:6887–6891. doi:10.1128/AEM.70.11.6887-6891.2004

Bouzianas DG (2010) Current and future medical approaches to combat the anthrax threat. J Med Chem 53:4305–4331. doi:10.1021/jm901024b

Chitlaru T, Altboum Z, Reuveny S, Shafferman A (2011) Progress and novel strategies in vaccine development and treatment of anthrax. Immunol Rev 239:221–236. doi:10.1111/j.1600-065X.2010.00969.x

Cote CK, Kaatz L, Reinhardt J, Bozue J, Tobery SA, Bassett AD, Sanz P, Darnell SC, Alem F, O'Brien AD, Welkos SL (2012) Characterization of a multi-component anthrax vaccine designed to target the initial stages of infection as well as toxaemia. J Med Microbiol 61:1380–1392. doi:10.1099/jmm.0.045393-0

Feinen B, Petrovsky N, Verma A, Merkel TJ (2014) Advax-adjuvanted recombinant protective antigen provides protection against inhalational anthrax that is further enhanced by addition of murabutide adjuvant. Clin Vaccine Immunol 21:580–586. doi:10.1128/CVI.00019-14

Fellows PF, Linscott MK, Ivins BE, Pitt ML, Rossi CA, Gibbs PH, Friedlander AM (2001) Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. Vaccine 19:3241–3247. doi:10.1016/S0264-410X(01)00021-4

Friedlander AM, Little SF (2009) Advances in the development of next-generation anthrax vaccines. Vaccine 27(Suppl 4):D28–D32. doi:10.1016/j.vaccine.2009.08.102

Gan J, Kendra K, Ricci M, Hank JA, Gillies SD, Sondel PM (1999) Specific enzyme-linked immunosorbent assays for quantitation of antibody-cytokine fusion proteins. Clin Diagn Lab Immunol 6:236–242

Hollmann A, Delfederico L, Miyoshi A, Disalvo EA, De Antoni G, Semorile L, Azevedo V (2010) S-layer proteins from lactobacilli as vaccine delivery systems. Intern Microbiol Res 2:30–43

Ilk N, Egelseer EM, Sleytr UB (2011) S-layer fusion proteins–construction principles and applications. Curr Opin Biotechnol 22:824–831. doi:10.1016/j.copbio.2011.05.510

Kaur M, Singh S, Bhatnagar R (2013) Anthrax vaccines: present status and future prospects. Expert Rev Vaccines 12:955–970. doi:10.1586/14760584.2013.814860

Mesnage S, Tosi-Couture E, Mock M, Gounon P, Fouet A (1997) Molecular characterization of the *Bacillus anthracis* main S-layer component: evidence that it is the major cell-associated antigen. Mol Microbiol 23:1147–1155. doi:10.1046/j.1365-2958.1999.01232.x

Mesnage S, Tosi-Couture E, Fouet A (1999) Production and cell surface anchoring of functional fusions between the SLH motifs of the *Bacillus anthracis* S-layer proteins and the *Bacillus subtilis* levansucrase. Mol Microbiol 31:927–936. doi:10.1046/j.1365-2958.1999.01232.x

Messner P, Steiner K, Zarschler K, Schaffer C (2008) S-layer nanoglycobiology of bacteria. Carbohydr Res 343:1934–1951. doi:10.1016/j.carres.2007.12.025

Mignot T, Mesnage S, Couture-Tosi E, Mock M, Fouet A (2002) Developmental switch of S-layer protein synthesis in *Bacillus anthracis*. Mol Microbiol 43:1615–1627. doi:10.1046/j.1365-2958.2002.02852.x

Mock M, Fouet A (2001) Anthrax. Annu Rev Microbiol 55:647–671. doi:10.1146/annurev.micro.55.1.647

Mohamadzadeh M, Duong T, Sandwick SJ, Hoover T, Klaenhammer TR (2009) Dendritic cell targeting of *Bacillus anthracis* protective antigen expressed by *Lactobacillus acidophilus* protects mice from lethal challenge. Proc Natl Acad Sci USA 106:4331–4336. doi:10.1073/pnas.0900029106

Nhan NT, de Valdivia EG, Gustavsson M, Hai TN, Larsson G (2011) Surface display of Salmonella epitopes in *Escherichia coli* and *Staphylococcus carnosus*. Microb Cell Fact. 10:22. doi:10.1186/1475-2859-10-22

Osorio M, Wu Y, Singh S, Merkel TJ, Bhattacharyya S, Blake MS, Kopecko DJ (2009) Anthrax protective antigen delivered by *Salmonella enterica serovar Typhi* Ty21a protects mice from a lethal anthrax spore challenge. Infect Immun 77:1475–1482. doi:10.1128/IAI.00828-08

Pomerantsev AP, Pomerantseva OM, Moayeri M, Fattah R, Tallant C, Leppla SH (2011) A *Bacillus anthracis* strain deleted for six proteases serves as an effective host for production of recombinant proteins. Protein Expr Purif 80:80–90. doi:10.1016/j.pep.2011.05.016

Schneewind O, Missiakas DM (2012) Protein secretion and surface display in Gram-positive bacteria. Philos Trans R Soc Lond B Biol Sci 367(1592):1123–1139. doi:10.1098/rstb.2011.0210

Shatalin KY, Neyfakh AA (2005) Efficient gene inactivation in *Bacillus anthracis*. FEMS Microbiol Lett 245:315–319. doi:10.1016/j.femsle.2005.03.029

Sleytr UB, Beveridge TJ (1999) Bacterial S-layers. Trends Microbiol 7:253–260. doi:10.1016/S0966-842X(99)01513-9

Sleytr UB, Huber C, Ilk N, Pum D, Schuster B, Egelseer EM (2007) S-layers as a tool kit for nanobiotechnological applications. FEMS Microbiol Lett 267:131–144. doi:10.1111/j.1574-6968.2006.00573.x

Tournier JN, Ulrich RG, Quesnel-Hellmann A, Mohamadzadeh M, Stiles BG (2009) Anthrax, toxins and vaccines: a 125-year journey targeting *Bacillus anthracis*. Expert Rev Anti Infect Ther 7:219–236. doi:10.1586/14787210.7.2.219

Wang YC, Jiang N, Zhan DW, Tao HX, Yuan SL, Wang P, Wang LC, Zhang ZS, Liu CJ (2011) Surface display of the 20-kDa N-terminal fragment of anthrax protective antigen based on attenuated recombinant *Bacillus anthracis*. World J Microbiol Biotechnol 27:2575–2581. doi:10.1007/s11274-011-0729-z

Wie SI, Andrews RE Jr, Hammock BD, Faust RM, Bulla LA Jr (1982) Enzyme-linked immunosorbent assays for detection and quantitation of the entomocidal parasporal crystalline protein of *Bacillus thuringiensis* subspp. kurstaki and israelensist. Appl Environ Microbiol 43:891–894