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ORIGINAL PAPER

Surface display of the 20-kDa N-terminal fragment of anthrax protective antigen based on attenuated recombinant *Bacillus anthracis*

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Abstract Extracellular antigen 1 (EA1), a major component of the *Bacillus anthracis* surface layer (S-layer), was used as a fusion partner for the expression of heterologous antigen. A recombinant *B. anthracis* strain was constructed by integrating a translational fusion harboring the DNA fragments encoding the cell wall–targeting domain of the S-layer protein EA1 and the 20-kDa N-terminal fragment of anthrax protective antigen (PA20) into the chromosome. A thermosensitive plasmid expressing Cre recombinase was introduced at a permissive temperature to remove the antibiotic marker. Cre recombinase action at the loxP sites excised the spectinomycin resistance cassette. The final derivative strains were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western blot analysis, and immunofluorescence analysis. PA20 was successfully expressed on the S-layer of the recombinant antibiotic marker-free strain. Guinea pigs were immunized with the attenuated recombinant *B. anthracis* strain, and the bacilli elicited a humoral response to PA20. This antibiotic marker-free strain and the correlative experiment method may have potential applications for the generation of a live attenuated anthrax vaccine.

Keywords *Bacillus anthracis* · Surface display · Cre–loxP system · Vaccine vehicle

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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 a bacterium (Sleytr and Beveridge 1999). The noncovalent interaction between the cell wall and S-layer proteins is very strong. The high level of expression of these proteins, together with their efficient binding to the cell wall, makes them an ideal system for studying cell surface anchoring and developing new expression systems (Mesnage et al. 1999a). *Bacillus anthracis* simultaneously synthesizes two S-layer proteins, namely, EA1 (extracellular antigen 1) and SAP (surface array protein). In the N-terminal region, each protein has three motifs of approximately 50 residues following the signal peptide, referred to as S-layer homology (SLH) motifs (Mesnage et al. 1997). These features allow the S-layer proteins to act as carrier proteins to display heterologous antigens on the cell surface (Mesnage et al. 1999b).

AP422, derived from the Chinese anthrax vaccine strain A16R, is an attenuated nontoxinogenic and nonencapsulated *B. anthracis* spore-forming strain. It could be used as a delivery vehicle to design a new type of anthrax spore vaccine.

In the present study, the 168-amino-acid 20-kDa N-terminal fragment of anthrax protective antigen (PA20), used as the target pathogen antigen, was expressed on the S-layer of the *B. anthracis* AP422 strain. The strategy developed may potentially generate an antibiotic marker-free live anthrax vaccine.

Materials and methods

Bacterial strains and growth conditions

All bacterial strains used in the present 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. *E. coli* strains were grown in Luria–Bertani (LB) broth and used as hosts for plasmid cloning. LB agar was also used to select transformants. *B. anthracis* strains were grown in brain heart infusion agar with 0.5% glycerol (BHIG; BD, USA) and LB media. Antibiotics (Merck, Germany) were added to the media 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*, transformation of *E. coli*, 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).

Plasmid construction

Table 1 shows the plasmids used in the present study, whereas Table 2 shows the oligodeoxynucleotide polymerase chain reaction (PCR) primers used.

1. pT-SLH-PA20::spc was used to construct the target fragment for the insertion of a portion of the *pagA* gene. A 1.4-kb spc^r fragment amplified from the plasmid pSET4s with two directly repeating loxP sites was inserted into the *T* vector pEASY-T1 (TransGen), and the *Sac*II and *Eco*RI sites were introduced. The fragments for SLH, PA20, and downstream *eag* were then amplified by PCR using the primers SLH-F and SLH-R, PA20-F and PA20F-R, and eag-d-F and eag-d-R, respectively. They were each subsequently inserted into the plasmid with spc^r according the design protocol (Fig. 1).
2. pMAD-SLH-PA20::spc was used for the insertion of the sequence encoding the PA20 fragment under the control of the *eag* gene. A 3.3-kb DNA fragment containing the SLH sequence of the *B. anthracis eag* gene, spc^r fragment, and downstream *eag* gene was obtained by digesting pT-SLH-PA20::spc with *Bam*HI

Table 1 Plasmids and strains used in this study

Plasmids and strains	Relevant characteristics	Source
Plasmids		
pT-lox P::spc	A spc^r with two directly repeated loxP sites of each end inserted the T- vector pEASY-T1	This work
pT-SLH-PA20::spc	T-Vector with fragment of slh-PA20-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-SLH-PA20::spc	pMAD with fragment of slh-pa20-loxP-spc-loxP	This work
pHY-cre	Expression vector of Cre recombinase	(Wang et al. 2009)
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
AP422::PA20	AP422, AP422, with partial eag gene replaced by the sequence encoding the PA20 fragment.	This work
E. coli strains		
Top10	*F-lacX74 recA1 araD139*Δ (*mcrA* Δ(*mrr-hsd RMS-mcrBC) φ80 lacZ*Δ *M15*Δ(*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*) (F′ *traD36 proAB lacI^q ZΔM15*)	Stratagene

CGMCC China general microbiological culture collection

Table 2 Primers used in this study

Primer	Sequence (5′ → 3′)	Restriction sites
SlhF	CGCGGATCCATGGCAAAGACTA	*Bam*HI
SlhR	CCAATGCATACCTGGTTTTCCATATTTTTT	*Nsi*I
PA20F	CCAATGCATGAAGTTAAACAGGAGAA	*Pst*I
PA20R	TCCCCGCGG TTATCGCTTTTTTCT	*Sac*II
eag-dF	CCGGAATTCAAAAATCTAGCTGTA	*Eco*RI
eag-dR	CCGCTCGAGAAGACTCATTCGCAAA	*Xho*I
eag-iF	TGAAAAACCTGACAGTTGTAAA	
eag-iR	CAAAAACGAATGCATTATCA	

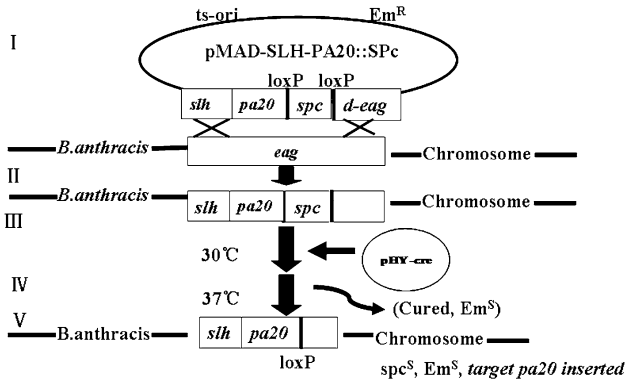

Fig. 1 Procedure for knockin of the *pa20* gene in *B. anthracis*

and *Xho*I. The fragment was then inserted into plasmid pMAD doubly digested with *Bam*HI and *Sal*I to create pMAD-SLH-PA20::spc. The constructed plasmids were identified by restriction endonuclease digestion and agarose gel electrophoresis.

Construction and isolation of mutants

B. anthracis mutants were constructed by replacing the coding sequences with the spc resistance cassette. The transformation and transformant selection of *B. anthracis* were performed as previously described (Arnaud et al. 2004; Pomerantsev et al. 2006). The pMAD-SLH-PA20::spc plasmid was introduced by electroporation into the AP422 strain of *B. anthracis*, and transformants were selected at 30°C on BHIG plates containing erythromycin and 50 μg/ml X-Gal (5-bromo-4-chloro-3-in-dolyl-β-D-galactopyranoside). Colonies were visible after 12–16 h. A single blue erythromycin-resistant colony was then transferred to an antibiotic-free liquid medium and incubated with shaking for 12 h at 42°C. After the fifth passage in the same medium, serial dilutions of this culture were plated on BHIG agar plates with spc resistance and incubated at 42°C overnight. After incubation, at least 20 white colonies were screened for spc resistance and erythromycin sensitivity. In some cases, additional passages were required to obtain the ideal phenotype. Colonies in which a double-crossover recombination event was suspected were validated by PCR analysis.

Plasmid pHY-Cre was electroporated into the isolates with selection for erythromycin resistance at 30°C to eliminate spc resistance. Erythromycin-resistant colonies were transferred to antibiotic-free agar and incubated at 37°C to eliminate pHY-Cre. Colonies were then patched to 3 separate agar plates containing erythromycin, spc, or no antibiotic, respectively. PCR and/or sequence analysis confirmed the presence of a single loxP site within the targeted gene of the mutants most sensitive to antibiotics.

Protein analysis

B. anthracis cells were grown in a BHIG medium for 14 h and washed once with phosphate-buffered saline (PBS). Bacterial pellets were resuspended in PBS with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer. The supernatants were boiled for 5 min and then centrifuged at 17,000*g*. The prepared samples were subsequently analyzed by SDS–PAGE in 12% gel. The separated proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, UK) using Bio-Rad Trans-Blot. The membrane was incubated in a blocking solution containing 5% fat-free milk for 1 h and then incubated with the monoclonal anti-PA antibodies (1:5,000 dilution; gifted by Professor Duan Qing). After washing, the membrane was incubated with rabbit anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP; Jackson, USA) at 1:5,000 dilutions. The bound antibody was detected using an HRP Western blot analysis system (TianGen). Another Western blot analysis was performed with the polyclonal anti-SLH domain antibodies (1:20,000 dilutions; this laboratory, unpublished data).

Immunofluorescence

Immunofluorescence analysis was mainly performed according to the protocol described by Mesnage et al.

(1999b). Bacteria grown on BHIG agar plates were washed with 20 mM PBS (pH 7.4), applied to a micro-coverglass 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 monoclonal anti-PA antibodies (1:100, v/v). Bacteria were washed twice with PBS–bovine serum albumin (1% BSA, wt/vol), and the primary antibodies were detected by incubating slides with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100, v/v; Jackson) 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

Flow cytometry analysis was performed to confirm the surface localization of the recombinant PA20 fusion proteins. An overnight culture of AP422, as the negative control, and the recombinant cells were harvested and washed thrice with PBS, then resuspended in PBS containing 1% skim milk and monoclonal anti-PA antibodies (1:200), and incubated on ice for 1.5 h. After washing thrice with PBS, the cells were incubated with FITC-conjugated goat anti-mouse IgG (1:100, v/v; Jackson) on ice for 1 h. The FITC-labeled cells were examined using a FACScan flow cytometer and with FACSCalibur (BD Biosciences, USA).

Immunization of guinea pigs

Female guinea pigs (obtained from the experimental center of the Academy of Military Sciences) weighing between 250 and 300 g were inoculated with vegetative cells via the intranasal route. The original strain AP422 was used as control. *Bacillus* species were grown overnight in a BHIG medium and diluted 1:1,000 in a fresh BHIG medium the following day. Cultures were grown at 37°C and shaken at 180 rpm. After 12 h of growth, the cultures were washed once with normal saline and suspended until the final concentration of 5×10^9 CFU/ml was obtained. For nasal feeding, guinea pigs were intranasally inoculated in the right naris with the suspension medium using a pipette delivering a single 50-µl volume. Booster doses were given on days 14 and 28, following the primary inoculation and in an identical manner.

All animal experiments were carried out in accordance with the regulations of the Academy of Military Sciences Animal Care and Use Committee.

Detection of specific IgG antibodies

An ELISA for the detection of IgG antibodies against PA20 was performed by adding serial twofold dilutions of sera from the immunized guinea pigs to a 96-well microtiter plate (Costar, USA) coated with the PA20 protein, which was expressed in *E. coli* BL21(DE3) (the present study, data not shown). The plates were coated with 500 ng protein/well of the recombinant PA20 in 50 mM $NaHCO_3$ buffer (pH 9.6). Rabbit anti-guinea pig IgG conjugated to alkaline phosphatase (Jackson) served as the second antibody. Color was developed using *o*-phenylenediamine as a chromogen and H_2O_2 as substrate. The absorbance was read at 492 nm.

Toxin neutralizing antibody assay

The LeTx neutralizing ability of guinea pig sera (at the last boost) was assessed on macrophage cell line RAW264.7. The RAW264.7 cells were seeded in a 96-well microtiter plate (Corning, USA) at 4–5 $\times$ 10^4 cells/well and maintained in an RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin for 16 h at 37°C under a 5% CO_2 atmosphere. Standards and serial dilutions of sera (triplicate wells for each dilution) were preincubated with LeTx (final concentrations: *rPA* 500 ng/ml, *LF* 300 ng/ml) in a humidified incubator set at 37°C under 5% CO_2 for 1 h. The medium was removed from the wells of the plates containing the cells and replaced with 100 µl/well of the samples preincubated with LeTx. After incubation for 4 h at 37°C under 5% CO_2, cell viability was assessed using a CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega, USA), following the manufacturer's instructions. Percentage of neutralization was calculated using the following formula: [Sample OD Value-LeTx Standard OD Value]/[Cells-only OD Value-LeTx Standard OD Value] $\times$ 100 (Kaur et al. 2009).

Statistical analysis

All statistical analysis procedures were performed with GraphPad Prism 4. Data are presented as mean ± SD. Statistical significance was tested using Student's paired *t* test, and $P < 0.05$ was considered statistically significant. The correlation coefficient (R) and two-tailed P value were calculated at the 95% confidence level.

Results

Construction of recombinant strains

Gene knockin was observed after electroporation of *B. anthracis* with the constructed plasmids. The procedure for *pa20* gene knockin is illustrated in Fig. 1. The shuttle vector pMAD carries a constitutively expressed transcriptional fusion, with the *bgaB* gene encoding a thermostable

β-galactosidase from *B. stearothermophilus*. Thus, the clones with whole plasmids show a blue phenotype on the X-Gal plates, whereas the disruption of the mutants without plasmid shows a white phenotype. Several white colonies were isolated on a BHIG medium at 37°C and verified for erythromycin sensitivity. The em^s spc^r white colonies with double-crossover events were identified as detailed in Materials and Methods to confirm *pa20* gene knockin. Chromosomal DNA was extracted from the clones and tested by PCR with the oligonucleotides EA1iF and PA20R (*data not shown*). All the white colonies on the X-Gal-containing plates contained the expected insertion and replacement in the *eag* locus after the selection process and were named AP422::PA20::spc. An AP422::PA20::spc clone was transformed with pHY-Cre to eliminate the spc cassette via Cre-mediated excision. This step was followed by curing the spc^s clones of the pHY-Cre plasmid by propagation at a restrictive temperature, producing the AP422::PA20 strain, which was sufficiently identified by PCR and sequencing analysis.

Characterization of the AP422::PA20 strain

The recombinant strain was grown in a BHIG medium. Samples from the crude extracts were subjected to SDS–PAGE and Western blot analysis using monoclonal antibodies against PA (Fig. 2). A strong signal corresponding to the size expected for the hybrid protein SLH-PA20 was detected in the crude extract of the recombinant strain (Fig. 2a, lane 2 approximately 42 kDa). The product was also recognized by anti-SLH antibodies (Fig. 2b, lane 2). A hybrid SLH-PA20 protein was hence successfully obtained in the present study. Immunofluorescence experiments showed that the fusion protein SLH-PA20, expressed by the recombinant strain, was located on the cell surface of the recombinant bacilli (Fig. 3b). In contrast, the control strain Ap422 was observed to express less green fluorescence (Fig. 3a).

FACS assays also provided evidence for surface localization. As shown in Fig. 4, under flow cytometry, a distinctive and homogenous increase in fluorescence was observed in AP422::PA cells as compared with the control strain (shift of histogram to the *right*, peak 2 in Fig. 4), indicating that the SLH-PA20 fusion protein is homogenously displayed onto the surface of the recombinant *bacilli*.

Immune responses of guinea pigs after nasal immunization with the live recombinant strains

The present study also investigated whether the surface with PA20 elicited a humoral response after immunizations. Guinea pigs were immunized on days 0, 14, and 28 by nasal immunization. After the 3 immunizations, each guinea pig was euthanized, and their blood was separately collected to analyze the effectiveness of strains delivering PA20 to the immune system. The antibody titers were determined by ELISA (Fig. 5). AP422::PA20 developed significantly high levels of PA20-specific serum IgG. The logarithmically transformed mean was approximately 3.180 ± 0.153, which was significantly different from that of the AP422 strain ($P < 0.001$). The results suggest that the vaccine-associated PA20 antigen of *B. anthracis* expressed on the surface of AP422 stimulates the immune response of experimental animals against specific antigens.

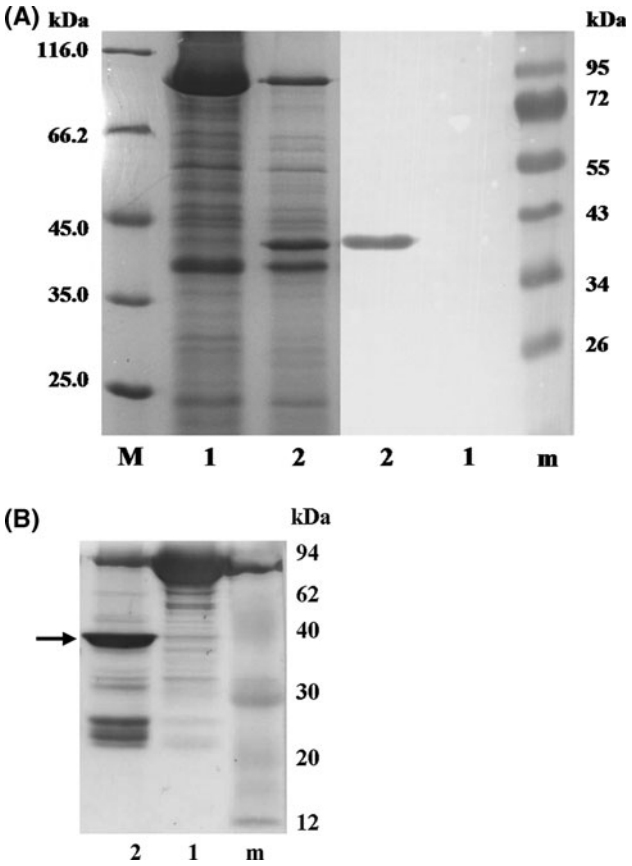

Fig. 2 Identification of mutant strains by SDS–PAGE and Western blot analysis. **a** SDS–PAGE and Western blot analysis using anti-PA monoclonal antibodies as the primary antibody. **b** Western blot analysis using anti-SLH domain polyclonal serum as the primary antibody. *Lane M* Protein marker, *lane m* stained protein marker, *lane 1* AP422, *lane 2* AP422::PA20

PA-specific antisera from guinea pigs were analyzed for LeTx neutralization as described in Materials and Methods. Only 27.59% protection from LeTx-associated cell death was observed when sera from the guinea pigs immunized with AP422::PA20 were diluted 10 times (Fig. 6), whereas AP422 exhibited neutralizing antibodies at the 12.11% level. Protection (*cell survival*) was not observed when sera from negative control guinea pigs were assessed for LeTx neutralizing activity.

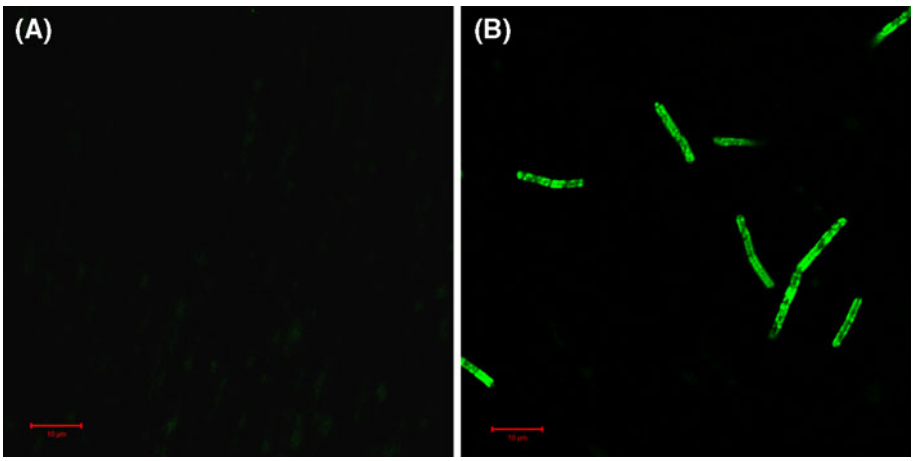

Fig. 3 Immunofluorescence microscopic analysis of whole AP422 (**a**) and AP422::PA20 cells (**b**). The *scale bar* represents 10 μm

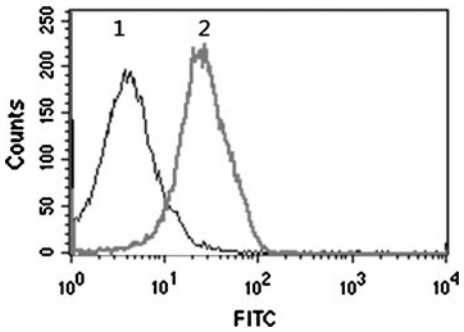

Fig. 4 FACS *histograms* of recombinant AP422::PA20 cells for determining the surface localization of the SLH-PA20 fusion protein. *1* AP422 cell (*used as negative control*), *2* AP422::PA20

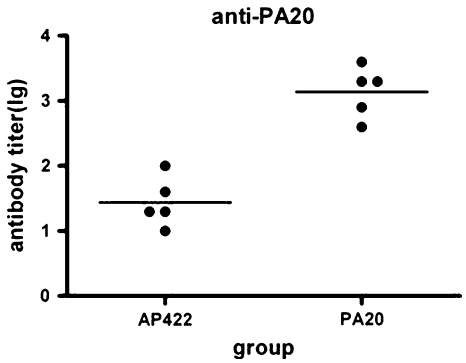

Fig. 5 Anti-PA20 IgG titer of guinea pig sera after three immunizations using recombinant strains. The logarithmically transformed mean of the anti-PA20 IgG titer of the AP422::PA20 strain was approximately 3.180 ± 0.153, whereas that of the AP422 strain was 1.440 ± 0.169

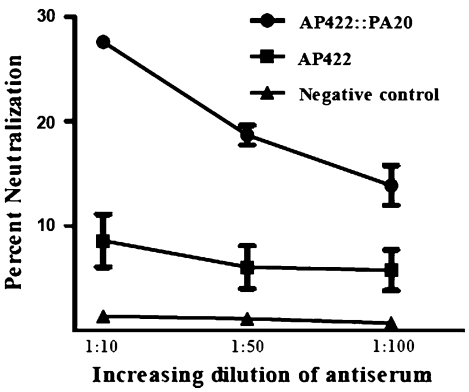

Fig. 6 Anthrax lethal toxin neutralization by PA-specific antibodies after immunization using recombinant strains

Discussion

The S-layer protein can be used as a surface display system to express the target protein and as a vaccine vehicle. Use of a homologous recombination method and the Cre–loxP recombinant system yielded the ideal AP422::PA20 strain, which can express the specified antigen on its surface without antibiotic resistance markers. Removal of the antibiotic resistance markers ensures that cell physiology will not be altered by known or unrecognized activities of the resistance genes or the antibiotic drugs themselves. Furthermore, the elimination of antibiotic resistance markers may increase the acceptance of recombinant strains for use in biotechnology applications, such as for live vector vaccines (Mohamadzadeh 2010; Tournier and Mohamadzadeh 2010). The present study shows that the S-layer surface display system may guide the development of a series of new live vaccines, especially anthrax vaccines.

Reason et al. (2008) demonstrated that the domain specificity of the isolated PA-specific human monoclonal antibodies from anthrax vaccine adsorbed-vaccinated donors was highly biased toward the 20-kDa N-terminal fragment of PA20, with 62% of the independently arising antibody clones reacting with determinants located on this PA fragment. This behavior suggests that the immunodominance of PA20-associated epitopes directly affects the efficacy of PA-based anthrax vaccines. Therefore, the PA20 was used in the present study as the surface expression target, and a recombinant strain was constructed. Guinea pigs elicited a humoral response after three immunizations

of vegetative cells. However, the data from the TNA indicate that the PA20-specific antibodies may not effectively neutralize toxin, which is consistent with previous research findings (Reason et al. 2008). These data indicate that the constructed strain in this work is not an ideal anthrax vaccine candidate. Nevertheless, if the whole PA protein is displayed on the S-layer, the new recombinant strain would become considerably valid according to research using PA20 as the analog. The present study therefore offers a novel strategy for designing anthrax vaccines using attenuated *B. anthracis*.

Attenuated strains of *B. anthracis* are suitable vehicles for vaccination. The promoters from the S-layer gene *eag* efficiently express the gene within the host once germination has occurred (Mesnage et al. 1999a, b, c), indicating that the spores of the AP422::PA20 could also be used for immunization when required. More importantly, numerous studies have shown that antigens can be delivered using spores simply as a vehicle to provide safe passage across the stomach barrier and that this route likely offers a more sophisticated one for vaccination because it ensures the protection of the encoded antigen (Duc et al. 2003; Duc and Cutting 2003; Aloni-Grinstein et al. 2005). Use of germinating spores could facilitate multiple rounds of cell replication and antigen expression because such spores have been shown to facilitate limited growth within the nasopharynx, lungs, and gastrointestinal tract (Hoa et al. 2001). In addition, the long-term storage properties of bacterial spores, coupled with simplified genetic manipulation and cost-effective manufacturing, make them particularly attractive vehicles for oral and intranasal vaccinations. Spore vaccines would thus be the focus of further research from our laboratory.

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