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METHODS AND PROTOCOLS

In silico analysis and recombinant expression of BamA protein as a universal vaccine against *Escherichia coli* in mice

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Abstract Colibacillosis, caused by pathogenic *Escherichia coli*, is a common disease in animals and human worldwide with extensive losses in breeding industry and with millions of people death annually. There is thus an urgent need for the development of universal vaccines against colibacillosis. In this study, the BamA protein was analyzed in silico for sequence homology, physicochemical properties, allergenic prediction, and epitopes prediction. The BamA protein (containing 286 amino acids) clusters in *E. coli* were retrieved in UniProtKB database, in which 81.7 % sequences were identical (Uniref entry A7ZHR7), and sequences with 94.82 % identity were above 93.4 %. Moreover, BamA was highly conserved among *Salmonella* and *Shigella* and has no allergenicity to mice and human. The epitopes of BamA were located principally in periplasm and extracellular domain. Surf_Ag_VNR domain (at position 448–810 aa) of BamA was expressed, purified, and then used for immunization of mice. Titers of the rBamA sera were 1:736,000 and 1:152,000 against rBamA and *E. coli* and over 1:27,000 against *Salmonella* and *Shigella*. Opsonophagocytosis result revealed that the rBamA sera strengthened the phagocytic activity of neutrophils against *E. coli*. The survival rate of mice vaccinated with rBamA and PBS was 80 and 20 %, respectively. These data indicated that BamA could serve as a promising universal vaccine candidate for the development of a protective subunit vaccine against bacterial infection. Thus, the above protocol would provide more feasible technical clues and choices for available control of pathogenic *E. coli*, *Salmonella*, and *Shigella*.

Keywords BamA · *E. coli* · Recombinant expression · Epitope · Vaccine

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Introduction

Escherichia coli, a pathogenic and opportunistic Gram-negative bacteria, can cause an infection in the intestines of livestock, poultry, and other animals, known as diarrhea, and may cause others urinary tract infection, sepsis, and meningitis in human (Ababneh et al. 2012; Lemaître et al. 2013; Tobias et al. 2015; Takeyama et al. 2015). The worldwide burden of these diseases is staggering, with significant economic losses in animal farms and with 840 million infections and 3,800,000 people death annually (Ron 2006; Mehla and Ramana 2016). Presently, these diseases caused by *E. coli* are mainly controlled by the use of antibiotics or vaccines. However, abuse of antibiotics in recent years has resulted in a significant upward trend in resistance (Grundt et al. 2012; Ababneh et al. 2012). A variety of vaccines against *E. coli* were developed to prevent colibacillosis and provided effective immune protections, including inactivated vaccine, attenuated vaccine, and subunit vaccine based on antigenic components of cells, such as adhesin and toxin (Holmgren et al. 2013; Lundgren et al. 2013; Lu et al. 2014; Sincock et al. 2016). In fact, however, vaccination is far from efficient against *E. coli* infections because of the multitudinous

serotypes of *E. coli* and unceasing emergence of resistant strains, which is why no *E. coli* vaccine has been widely used worldwide (Mehla and Ramana 2016). Hitherto, there are no licensed vaccines available for enterotoxigenic *E. coli* (Mehla and Ramana 2016). In China, only several inactivated vaccines are currently available to prevent or treat colibacillosis in piglets, sheep, chicken, ducks, and rabbits. However, these inactivated vaccines with single specific serotype showed limited protection against *E. coli* infection; by contrast, universal vaccines have several advantages over them, including convenience, cost-effectiveness, and cross-protection efficiency (Guan et al. 2015; Wang et al. 2015). The development of versatile vaccines is an urgent need to control and prevent against pathogenic *E. coli* infection especially in China while avoiding the use of antibiotics.

Outer membrane proteins were widely distributed in Gram-negative bacteria. Surface-exposed outer membrane proteins are primary targets recognized and attacked by the host immune system; therefore, outer membrane proteins constitute potential vaccine candidates. Due to their exposed epitopes on the cell surface, several highly conserved outer membrane proteins in *E. coli* such as OmpA, OmpC, and OmpF have been reported to offer good immune protective effects in mice (Hu et al. 2013; Hsieh et al. 2014; Guan et al. 2015; Yu et al. 2008; Liu et al. 2012; Wang et al. 2015). The outer membrane protein BamA in *E. coli* is an essential component of the hetero-oligomeric machinery that mediates β-barrel outer membrane protein assembly (Bennion et al. 2010). BamA belongs to the Omp85 family of proteins, which are major antigenic and immunogenic proteins expressed by most Gram-negative pathogenic bacteria (Su et al. 2010). To our knowledge, there is no report on the application of BamA vaccine against colibacillosis and other bacterial diseases.

In this study, physicochemical properties, allergenicity, and epitopes of the BamA protein in *E. coli* CVCC 1515 were analyzed by bioinformics software. The *bamA* gene was cloned and expressed in *E. coli* BL21 (DE3) with a 6×His fusion tag. Immune response and protective efficacy of the recombinant BamA protein (rBamA) were evaluated for a universal vaccine candidate, and we hope it can be validated and served as a reference for much more similar work.

Materials and methods

Bacterial strains and mice growth conditions

The strains of *E. coli* CVCC 1515, *E. coli* CVCC 195, *Salmonella choleraesuis* CVCC 503, *Salmonella enteritidis* CVCC 3377, and *Salmonella pullorum* CVCC 1802 were purchased from China Veterinary Culture Collection Center (CVCC) (Beijing, China). The strains of *E. coli* CICC 21530 (serotype O157:H7), *Salmonella typhimurium* CICC 22596, and *Pseudomonas aeruginosa* CICC10419, CICC 21625, CICC 21636, and CICC 22630 were purchased from the China Center of Industrial Culture Collection (CICC) (Beijing, China). The *S. enteritidis* CMCC (B) 50336, *Shigella dysenteriae* CMCC (B) 51252, and CMCC (B) 51571 strains were purchased from the National Center for Medical Culture Collection (CMCC) (Beijing, China). The *E. coli* DH5α and BL21 (DE3) strains were purchased from TransGen Biotech, Inc. (Beijing, China). All strains were cultured on Luria Bertani (LB) at 37 °C.

Specific pathogen-free (SPF) female BALB/c mice (6~8 weeks old) were purchased from Vital River Laboratories (VRL, Beijing). Mice were housed in appropriate conventional animal care facilities and handled strictly according to international guidelines required for animal experiments.

Sequence homology analysis of BamA

The amino acid sequence of the BamA protein in *E. coli* was retrieved from Uniport database (http://www.uniprot.org/), with gene name "*BamA*" and organism "*Escherichia coli*." These sequences of BamA were aligned by the "Align" tool (http://www.uniprot.org/align/). Representative sequences of BamA in *E. coli* (UniRef entry B7MP37, T8KAF0, N2JZB7, and A7ZHR7) were also aligned with those of BamA in *Salmonella* (UniRef entry Q5PD65, Q8ZRP0, S5GQW9, B5RHG2, B5FJ24), *Shigella* (UniRef entry F3WE14, I6DUK6, Q32JT2), and *Pseudomonas* (UniRef entry S6J182, S6MPY6, A0A038GD14, A0A0D6GGR7), respectively, with CLUSTALO program. Phylogenetic tree was constructed by using the MEGA 6 software.

The genomic DNA from the *E. coli* CVCC 1515 strain was extracted with a TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. Primer pairs for the *bamA* gene in *E. coli* CVCC 1515 were designed based on representative sequence (Uniref entry A7ZHR7). After PCR amplification, the PCR products of *bamA* were sequenced and compared with the representative BamA protein sequence with blastx in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastn).

Physicochemical properties analysis of BamA

The BamA protein (Uniref entry A7ZHR7) was analyzed as a model, and its conserved domains were analyzed in NCBI (Marchler-Bauer et al. 2015). Physical and chemical parameters of this protein were calculated with ProtParam tool (http://web.expasy.org/protparam/). Solubility of BamA overexpressed in *E. coli* was predicted using a

software (http://biotech.ou.edu/#rt by University of Oklahoma) (Diaz et al. 2010).

Allergenicity prediction of the BamA protein

Allergenicity of BamA to mice and human was predicted with the AlgPred tool (http://www.imtech.res.in/raghava/algpred/submission.html) by performing BLAST search against allergen representative peptides and by mapping of IgE epitope (Saha and Raghava 2006).

B cell epitope prediction of the BamA protein

Linear B cell epitopes were predicted based on sequence properties with threshold 0.35 (http://tools.immuneepitope.org/bcell/) (Larsen et al. 2006; Ponomarenko and Bourne 2007). Discontinuous B cell epitopes were predicted by DiscoTope 2.0 (http://tools.immuneepitope.org/stools/discotope/discotope.do) which was based on 3D structures of proteins in PDB format (Kringelum et al. 2012). Prediction threshold was set at -3.1. PDB id of BamA protein were 3EFC (21–410 aa) and 4C4V (344–810 aa) (Gatzeva-Topalova et al. 2008; Albrecht et al. 2014).

Expression and purification of "Surf_Ag_VNR" domain of the BamA protein

According to the bioinformation analysis result of the BamA protein, the complementary DNA (cDNA) of "Bac_surface_Ag" region (at position 448–810 aa) of BamA in *E. coli* CVCC1515 (GenBank KP057879) was amplified by primers of F-EcoRI: 5′-GAATTCAATTG GTTAGGTACAGGTTATGC-3′ with the *Eco*RI site and R-NotI: GCGGCCGCCCAGGTTTTGCCGATGTTGAACT with the *Not*I site.

The *bamA* PCR product was inserted into the pET28a expression vector. The recombinant plasmids were then transformed into *E. coli* BL21 (DE3). The His-tagged BamA protein in BL21 was expressed using a modified auto-induction method (Studier 2005). Briefly, monoclonal strains were inoculated in LB at 37 °C on a platform shaker at a speed of 250 rpm until an optical density at 600 nm (OD_{600}) of 0.60 and then transplanted to ZYM-5052 autoinduction media with 1 % inoculum density. The strains were cultured at 37 °C on a platform shaker at a speed of 250 rpm for 24 h.

Purification and refolding of the recombinant outer membrane protein was improved based on the previous protocol (Saleem et al. 2012). Briefly, after the fusion protein was sufficiently expressed, the bacteria were pelleted and resuspended in lysis buffer (50 mM Tris–HCl buffer, pH 7.9, containing 5 mg of lysozyme per gram of cell paste and 5 μl of DNase I type IV stock per gram of cell paste) with 8 ml buffer per gram wet weight of cell paste. The cells were disrupted in a probe ultrasonicator. Inclusion bodies (IBs) were precipitated by centrifugation at 14,000×*g* for 20 min at 4 °C and washed twice in 50 ml of 50 mM Tris–HCl buffer (pH 7.9, containing 1.5 % (*v*/*v*) lauryl dimethyl amine oxide (LDAO)) for each 1~1.5 g wet weight. After that, the IBs were precipitated and dissolved in denaturing buffer (10 mM Tris–HCl buffer, pH 7.5, containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 8 M urea). The IB solution was centrifuged at 14,000×*g* for 20 min to remove any undissolved material and was added to refolding buffer (20 mM Tris–HCl buffer, pH 7.9, containing 1 M NaCl and 5 % (*v*/*v*) LDAO) dropwise with rapid stirring to produce a final 1:1 volume ratio. The solution was dialyzed at 4 °C against two changes of 4 l of dialysis buffer (20 mM Tris–HCl buffer, pH 7.9, containing 0.5 M NaCl and 0.1 % (*v*/*v*) LDAO) every 6 h for refolding.

The refolding 6×His-Tag fusion protein was purified using a Ni^{2+}-nitriloacetate (NTA) super flow resin column (QIAGEN, Germany) with equilibration buffer (20 mM Tris–HCl buffer, pH 7.9, containing 0.5 M NaCl, 0.1 % (*v*/*v*) LDAO and 40 mM imidazole) and elution buffer (20 mM Tris–HCl buffer, pH 7.4, containing 0.5 M NaCl, 0.1 % (*v*/*v*) LDAO and 500 mM imidazole) according to the manufacturer's instructions. Then the eluted recombinant protein was desalted using a HiPrep 26/10 desalting column with desalination buffer (20 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl and 0.1 % (*v*/*v*) LDAO). All proteins were determined by 12 % sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS–PAGE). The protein was then lyophilized with the ALPHA 1-2 LD plus freeze dryer (Christ, Germany) and kept in −20 °C.

Western blotting analysis of rBamA

SDS–PAGE was performed by running the gel for 120 min at 80 V. The rBamA protein was transferred to the PVDF membrane as described previously (Li et al. 2014). Briefly, the PVDF membrane was blocked overnight with 5 % BSA in TBST (25 mM Tris, 150 mM NaCl, and 0.05 % (*v*/*v*) Tween-20, pH 7.4) at 4 °C. After washing three times with TBST, the membrane was incubated with the rBamA sera (1:5000) for 2 h at room temperature (RT). After washing with TBST, the membrane was incubated with secondary antibodies (Beijing CWBIO Co., Ltd.) at a dilution of 1:5000 for 2 h at RT. The membrane was then washed, and the bands were stained using BCIP/NBT Solution (Beijing CWBIO Co., Ltd.) as substrate.

Immunization protocols

The lyophilized protein was resuspended in sterile PBS to obtain a concentration of 1.0 mg/ml. Twenty BALB/c mice were immunized with the purified rBamA protein on day 0, day 21 and day 35 (Guan et al. 2015). For the first immunization, antigen solution (25 μl) was mixed with complete Freund's

adjuvant (Sigma-Aldrich, Inc.) (25 μl) and PBS (50 μl). Mice were vaccinated with 100 μl antigen mixture per mouse by hypodermic injection.

For the second immunization, antigen mixture was composed of antigen solution (25 μl), incomplete Freund's adjuvant (Sigma-Aldrich, Inc.) (25 μl), and PBS (50 μl). Mice were intraperitoneally injected with 100 μl antigen mixture per mouse (BamA group). Mice immunized with PBS were used as the control group (PBS group). The third booster immunization was carried out with the same procedure. All mice were housed individually in ventilated cages (Suzhou Fengshi Laboratory Animal Equipment Co., Ltd., Suzhou) and monitored daily. Cages were changed once per week. The mice were bled on day 1, day 25 and day 39 from the tail vein. Sera were stored at −20 °C until used.

BamA detection by the enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well plates were coated with 0.2 μg/well BamA protein in 100 μl of coating buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6) by overnight incubation at 4 °C. The plates were washed four times with PBST (PBS containing 0.05 % Tween-20) and then blocked with 5 % BSA in PBST at 37 °C for 2 h. After washing with PBST four times, the plates were added with serial dilutions of mice serum and incubated for 1.5 h at 37 °C and washed as above. HRP-conjugated goat anti-mouse IgG (diluted with 1:5000) was added into the plates with 100 μl/well and incubated for 30 min at 37 °C. After that, the color was developed with 3,3′,5,5′,-tetramethylbenzidine (TMB) for 20 min at 37 °C. 2 M H_2SO_4 (50 μl/well) was used to stop the reaction. The absorbance of each well was read at 450 nm by a microplate reader (Perlong Medical, Beijing). The $OD_{450\ nm}$ (test group)/$OD_{450\ nm}$ (negative control) ratio ≥2.1 was considered as a positive result.

Bacterial cell detection by ELISA

Ninety-six-well plates were added with 150 μl 0.1 M $NaHCO_3$ plus 2.5 % glutaraldehyde, incubated for 1 h at 37 °C, and washed four times with sterile water. The plates were then coated with 10^7 CFU/100 μl bacterial cells per well and incubated at 37 °C until dry. Subsequent steps from antigen blocking were carried out in accordance with the above procedure.

Opsonophagocytosis assay

Mice neutrophils were isolated from peritoneal fluid using a previously described protocol (Guan et al. 2015). The concentration of neutrophils was adjusted to 4×10^6 cells/ml. The *E. coli* CVCC 1515 strain was cultured to logarithmic phase and adjusted to 4×10^4 CFU/ml. For each sample, 400 μl of bacterium suspension was mixed with mouse serum at the ratio of 4:1. The mixture was incubated at 30 °C for 30 min. Five hundred microliters of neutrophils suspension and 100 μl of baby rabbit complement (Cedarlane, Homby, Ontario, Canada) were added into the mixture and incubated at 30 °C for 1 h. After incubation, neutrophils were lysed by adding sterile water into the mixture. The mixture was then serially diluted for plate counting. The survival rate of bacterial cells was calculated as the ratio between colony in each group and the blank group.

Challenge assay

A lethal dose of 50 % (LD_{50}) was determined by the previous method (Guan et al. 2015). Based on this, 14 days after the second immunization, ten mice from each group were injected intraperitoneally with 100 μl (1×10^9 CFU/ml) log-phase *E. coli* CVCC 1515. Mortality was recorded for the next 180 h.

Statistical analysis

The SPSS software (version 22) was used for all statistical analyses. One-way repeated analysis of variance (ANOVA) and the Mann–Whitney rank test were used to evaluate differences between groups. Differences were considered significant at $p < 0.05$.

Results

Homology and phylogenesis analysis

The BamA protein clusters in *E. coli* were obtained in UniProtKB database, in which 81.7 % sequences were identical (Uniref entry A7ZHR7), and sequences with 94.82 % similarity were above 93.4 % (Fig. 1a, b).

The BamA protein from *E. coli* shares 90.99, 99.14, and 31.92 % identity with that from *Salmonella*, *Shigella*, and *Pseudomonas* strains, respectively. The results showed that the BamA protein is highly conserved among *E. coli*, *Salmonella*, and *Shigella*, but shares low homology with that of *Pseudomonas*. Phylogenetic tree also indicated that *Pseudomonas* has a larger genetic distance than the other three bacteria. The BamA sequence from *E. coli* (Uniref entry A7ZHR7) was used as a model in the following analysis. The *bamA* gene in *E. coli* CVCC 1515 was sequenced and aligned by BLAST with representative sequences in *E. coli*. The result showed that the BamA protein sequence was identical to that of A7ZHR7.

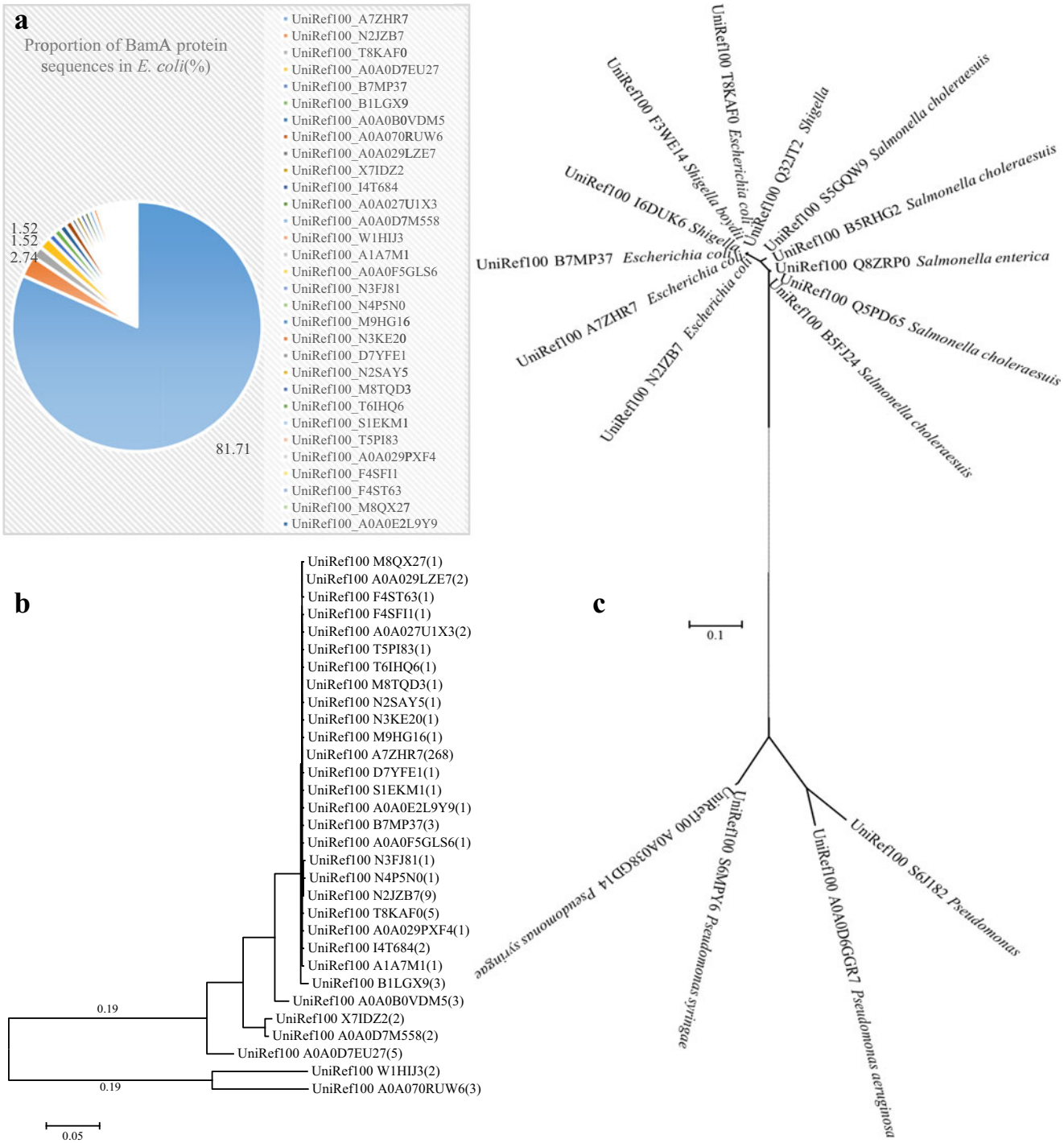

Fig. 1 Homology and phylogenesis analysis of the BamA protein from *E. coli* CVCC 1515. **a** Proportion of the BamA protein sequences in *E. coli* (%). **b** Phylogenesis analysis of the BamA protein in *E. coli*. **c** Phylogenesis analysis of the BamA protein in *E. coli*, *Salmonella*, *Shigella*, and *Pseudomonas*

Structure and physicochemical properties analysis of the BamA protein

Six conserved domains at positions 24–91 aa, 92–172 aa, 266–344 aa, 175–263 aa, 347–421 aa and 448–810 aa were existed in the BamA protein (Fig. 2). The first domain at positions 24–91 aa belonged to surface antigen with variable number repeats (Surf_Ag_VNR), and the fragment from positions 448 to 810 aa belonged to bacterial surface antigens (Bac_surface_Ag). The ProtParam results showed that the outer membrane protein mainly included β-strand, helix, and turn. The fragment of position from 1 to 20 aa was a signal

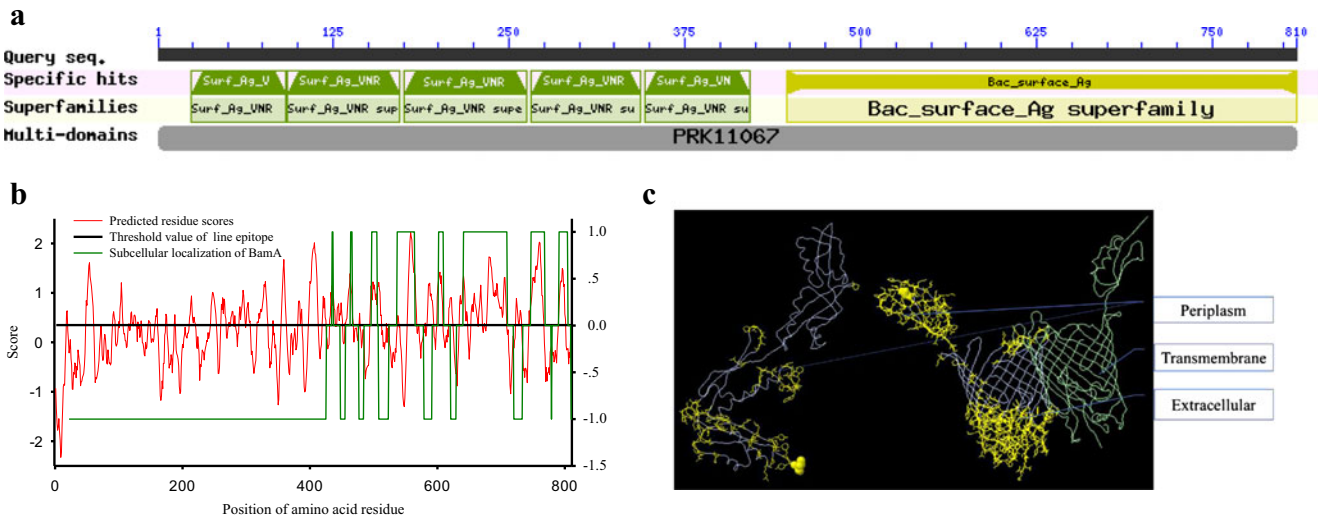

Fig. 2 Structure, position of conserved domains, and subcellular location and B cell epitope prediction of the BamA protein. **a** Structure and position of conserved domains. **b** Discontinuous B cell epitope. **c** Subcellular location and linear B cell epitope. *Yellow color* represents epitopes

peptide, and the fragment of position from 21 to 810 aa was a mature transmembrane protein which containing helix, β-strand, and turn. Figure 2a displays the subcellular location of BamA, the position of 21–424 aa was in periplasm, and eight loops of extracellular domain were distributed in position of 434–810 aa. Due to containing 56.65 % hydrophobic amino acids, BamA had a 0.0 % chance of solubility when being overexpressed in *E. coli*.

Allergenic prediction of the BamA protein

Allergenicity of BamA was predicted based on two algorithms, blasting against allergen representative peptides and mapping IgE epitope. The result showed that no hits were found, indicating that the BamA protein has no allergenicity to mice and human.

B cell epitope prediction of the BamA protein

Linear B cell epitope was predicted by BepiPred based on a combination of a hidden Markov model and a propensity scale method (Larsen et al. 2006). The residues with scores above the threshold (default 0.35) are predicted to be part of an epitope. As shown in Fig. 2b, linear epitopes were composed of more than seven amino acid residues, which existed in the periplasm (14 epitopes) and extracellular domain (14 epitopes). Discontinuous epitopes were predicted by DiscoTope 2.0 (Kringelum et al. 2012), which was a structure-based prediction tool. The results showed that discontinuous epitopes were located principally in the periplasm and extracellular domain (Fig. 2c).

Expression, purification, and Western blotting analysis of the BamA protein

The *bamA* gene was cloned into a pET-28a vector, and the recombinant plasmid pET-28a-BamA was transformed into BL21 (DE3) cells for auto-induction expression. The BamA protein was successfully expressed in *E. coli* BL21 with an N-terminal 6×His tag. After purification, the rBamA protein was estimated to be approximately 41 kDa by SDS–PAGE with the purity of 93.5 % (Fig. 3a). One main band with the size of 41 kDa was observed in Western blotting (Fig. 3b), which was consistent with the BamA expression gels. It indicated that the

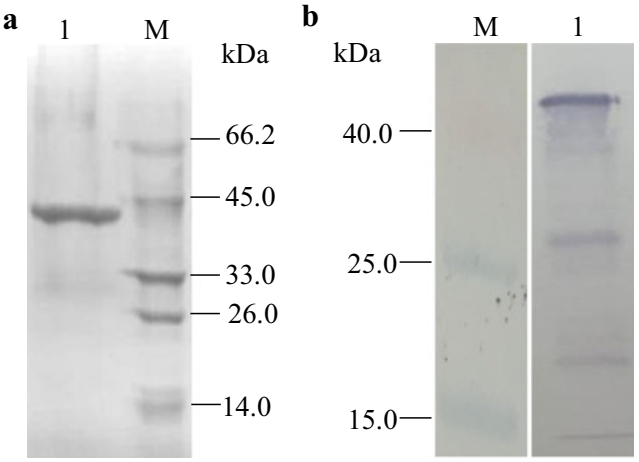

Fig. 3 Purification and Western blotting analysis of the BamA protein. **a** Purification of the BamA protein. *Lane 1*, the BamA protein; *lane M*, protein marker. **b** Western blotting analysis of the BamA protein. *Lane M*, protein marker; *lane 1*, the BamA protein

anti-BamA serum mainly bound with BamA. These results taken together suggest that BamA is an immunogenic protein.

Strong antibody induced by immunization with rBamA

Sera were collected from mice on days 0, 5, 25, and 39, and tested by ELISA using the rBamA protein and *E. coli* CVCC 1515 as antigens. As shown in Fig. 4a, titers of the rBamA sera against rBamA were increased from 1:90 to 1:496,000 or to 1:736,000, respectively, after the second and third immunizations with rBamA. Titers of the rBamA sera against *E. coli* CVCC 1515 raised from 1:50 to 1: 48,000 or to 1:152,000, respectively, after the second and third immunizations. Titers of the PBS sera against rBamA were lower than 1:100. It indicated that rBamA could induce high titers of antibody.

Cross reactivity and phagocytosis of the rBamA sera in vitro

Cross reactivity of the rBamA sera was measured by the whole cell ELISA assay against the *E. coli*, *Shigella*, *Salmonella*, and *Pseudomonas* species. Ratio of test group and negative control group greater than 2.1 was considered as positive. As shown in Fig. 4b, the rBamA sera had high cross reactivity with *E. coli*, *Shigella*, and *Salmonella* strains, but no reactivity with all *Pseudomonas* strains, indicating that the BamA sequence was highly conserved among *E. coli*, *Shigella*, and *Salmonella*.

The neutrophils of mice were mixed with the *E. coli* CVCC 1515 strain and baby rabbit complement. After incubation, the mixture was serially diluted for plate counting. After incubation for 30 min, the ratio of viable bacterial cells in PBS sera and rBamA sera decreased to 81.74 % ($p > 0.05$) and 41.68 % ($p < 0.01$), respectively

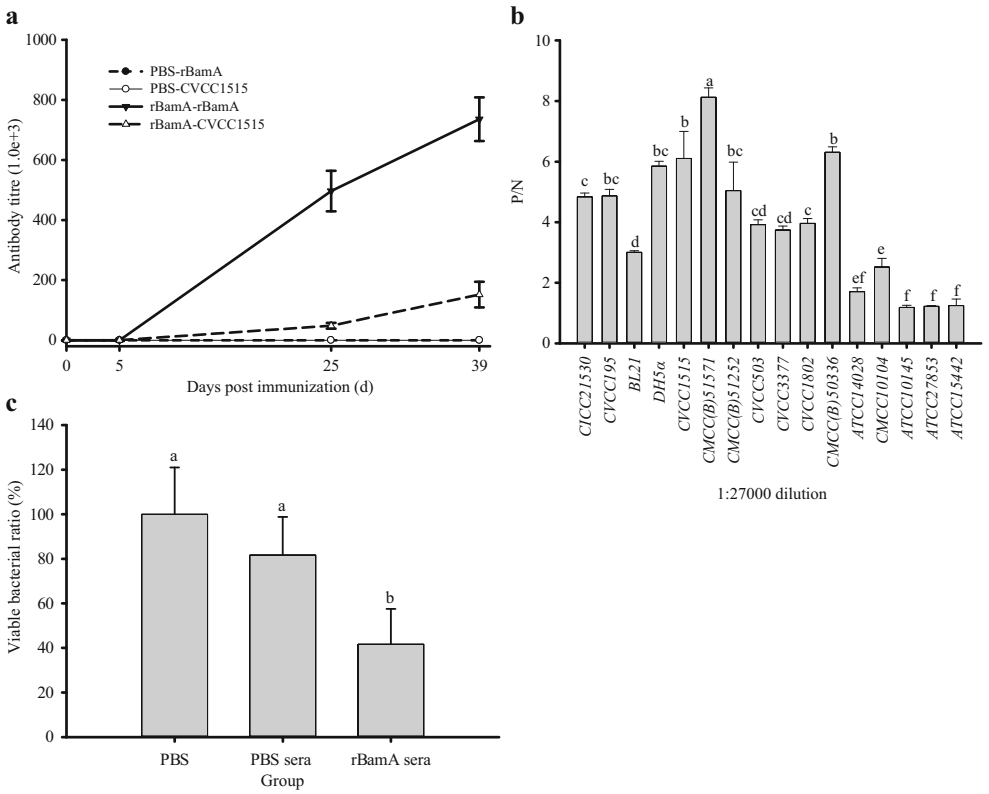

Fig. 4 Antibody titers and phagocytosis of rBamA. Mice were vaccinated with rBamA or PBS. Serum samples were collected at days 0, 5, 25, and 39, respectively, and antibody titers were measured by ELISA using the purified rBamA as antigen. **a** Antibody titer in different groups post-vaccination. *PBS-rBamA* the antibody titer of PBS vaccination group against rBamA, *rBamA-rBamA* the antibody titer of rBamA vaccination group against rBamA, *PBS-CVCC1515* the antibody titer of PBS vaccination group against *E. coli* CVCC 1515, *rBamA-CVCC1515* the antibody titer of rBamA vaccination group against *E. coli* CVCC 1515. **b** Cross-reaction properties of the anti-rBamA sera (titer of 1:27,000) against different bacteria. *P*/*N* value of different bacteria. Statistical deviations are indicated by a *lowercase letters* ($p < 0.05$). **c** Phagocytosis of the rBamA sera in vitro. Colony counts of different groups in phagocytosis were determined by plate count. *E. coli* strain CVCC 1515 was incubated with anti-sera of PBS vaccination group (PBS sera group), anti-sera of rBamA vaccination group (rBamA sera group) and PBS (PBS group). Statistical significance ($p < 0.05$) is indicated by a *lowercase letter*

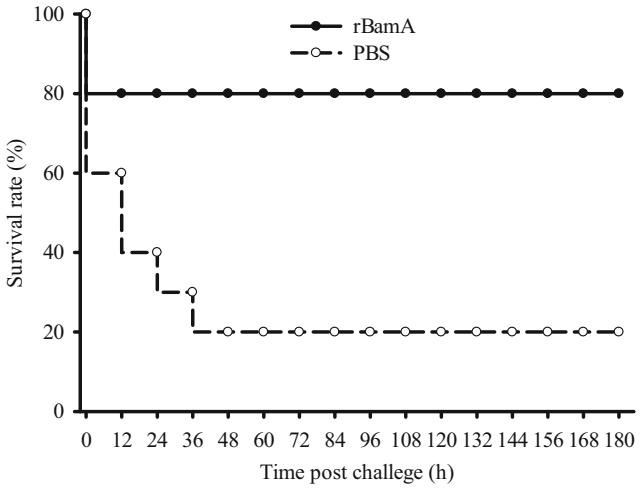

Fig. 5 Survival percent of the mice immunized with rBamA or PBS. Mice immunized with rBamA or PBS (control) were intraperitoneally injected with log-phase *E. coli* CVCC 1515 (10^8 CFU) and observed for 180 h after challenge

(Fig. 4c), suggesting that the rBamA sera enhanced the phagocytic activity of neutrophils against *E. coli* in vitro.

Protective efficacy of the rBamA sera against *E. coli* in vivo

Fourteen days after the third immunization, mice were challenged with 100 μl (10^9 CFU/ml) *E. coli* CVCC 1515. As shown in Fig. 5, a significant difference was observed in survival rate of mice immunized with rBamA and PBS. After 180 h post-challenge, the survival rate of mice in the rBamA group was 80 %, higher than that in the PBS group (20 %), which demonstrated that the rBamA protein provided a significant level of protection against challenge with *E. coli* in a mouse model.

Discussion

As a novel universal protein vaccine against Gram-negative bacteria, it should have the following several important features: firstly, it should be non-toxic and non-allergic to human and animals. Secondly, its antigenic component should be located on the surface of pathogenic bacteria (Foster et al. 2014). Thirdly, it should extensively distribute and be highly conserved in Gram-negative bacteria (Hubert et al. 2013). Finally, it should have strong antigenic specificity and immunoreactivity. To date, several outer membrane proteins of *E. coli* have been identified as potential vaccine candidates against colibacillosis because members of this family of proteins possess the above-mentioned characteristics (Khushiramani et al., 2007; Liu et al. 2012; Guan et al. 2015; Wang et al. 2015). These merits or advantages from novel universal protein vaccine are obvious with regard to practical importance when compared with the commercial inactivated vaccines during epidemic prevention by immunization in animal husbandry in China. This is why we have tried to exploit OmpA (Guan et al. 2015), OmpC (Wang et al. 2015) in previous work, and BamA in this work as the novel universal protein vaccine candidates against *E. coli*.

In the light of our previous experience in *E. coli* vaccine development (Guan et al. 2015; Wang et al. 2015), it seems that construction of universal vaccines based on outer membrane proteins against different pathogenic bacteria is possible. In present study, the BamA protein was firstly analyzed for conservative property, physicochemical properties, structure, and immunogenicity in silico. Sequence homology analysis revealed that the BamA protein from *E. coli* CVCC 1515 was highly conserved among *E. coli*, *Salmonella*, and *Shigella*, except for *Pseudomonas* species (Fig. 1). It suggests that antibody induced by BamA may have a high affinity for *E. coli*, *Salmonella*, and *Shigella* species, but not for *Pseudomonas* strains. It means that the antigen could be targeted as a universal vaccine candidate in controlling diseases caused by *E. coli*, *Salmonella*, and *Shigella* species.

In general, the protein regions predicted to be present in the periplasm would be more likely to be involved in immunogenicity than the regions present within the inner membrane of the bacteria (Harland et al. 2007). Immunodominance of antigens was determined by location of this epitope in antigen molecules (Hiszczyńska-Sawicka et al. 2014). Epitope prediction was mainly based on primary structure such as hydrophilicity, accessibility, antigenicity, and flexibility, or secondary structure such as α-helix and β-turn (Ferrante 2013; Yao et al. 2013). In this study, the predicted epitopes at position 448–810 aa in BamA were distributed in periplasm and extracellular domains of *E. coli* (Fig. 2). The cDNA sequence was cloned and expressed at a size of approximately 41 kDa by SDS–PAGE (Fig. 3a). Immunoassay results showed that the rBamA protein is immunogenic in mice. Titers of the rBamA sera against rBamA were higher than those of against *E. coli* (Fig. 4a). Cross-reactivity data further revealed that the rBamA sera reacted with the whole cell of *E. coli*, *Shigella*, and *Salmonella* species, but did not with *Pseudomonas* strains (Fig. 4b), which is in good agreement with above-predicated results (Fig. 1). The results suggest that the rBamA antigen could be targeted as a universal vaccine candidate in controlling diseases caused by *E. coli*, *Salmonella*, and *Shigella* species. Colony count of *E. coli* in rBamA group (41.68 %) remarkably decreased compared with that in PBS group (81.74 %), revealing that phagocytosis of neutrophils was significantly enhanced in the presence of specific rBamA antibody. Moreover, mice vaccinated with rBamA were well protected when challenged with *E. coli* CVCC 1515, and the survival rate of mice was up to 80 % (Fig. 5), whereas the majority PBS-immunized mice succumbed within 36 h after infection (20 % survival rate), indicating that the potent

immune protection was provided by rBamA immunization against *E. coli* infection. Moreover, it was speculated that vaccination with rBamA maybe induce a humoral immune response capable of recognizing the native membrane protein located on the *E. coli* cell surface, in accordance with the viewpoint proposed by Su et al. (Su et al. 2010).

In conclusion, the BamA protein was highly conserved among *E. coli*, *Salmonella*, and *Shigella*, except for *Pseudomonas*. Linear epitopes and discontinuous B cell epitopes were distributed in periplasm and extracellular domains of *E. coli*. After expression and purification, rBamA was used for immunization of mice. The rBamA sera had a high affinity to rBamA and *E. coli*, a strong cross reactivity with *Salmonella* and *Shigella* strains, and enhanced the phagocytic activity of neutrophils. Mice immunized with rBamA antigens were potently protected against lethal challenge with *E. coli*. We believe the technical protocol based on this work would provide the more feasible solutions with advantage of performance-cost ratio over other products during struggle for control of key pathogenic *E. coli*, *Salmonella*, and *Shigella* in practice of animal husbandry, and thus the protocol's methodology would be much more important than this universal vaccine itself when applied to similar work.

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Compliance with ethical standards

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

Ethical approval The animal protocol for the present study was approved by the Animal Care and Use Committee of the Feed Research Institute, Chinese Academy of Agricultural Sciences (Beijing, China), and all mice involved were cared for in accordance with the institutional guidelines from the above committee.

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