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Protection against *Acinetobacter baumannii* infection via its functional deprivation of biofilm associated protein (Bap)

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ABSTRACT

Acinetobacter baumannii, a major nosocomial pathogen, has remarkable capacity to acquire antimicrobial resistance attributable to its biofilm formation ability. Biofilm associated protein (Bap), a specific cell surface protein, is directly involved in biofilm formation by *A. baumannii* and plays a major role in bacterial infectious processes. In the present study we cloned, expressed and purified a 371 amino acid subunit of Bap. Mice were immunized using recombinant Bap subunit. They were then challenged with *A. baumannii* to evaluate the immunogenicity and protectivity of Bap subunit. Humoral immune response to Bap was determined by ELISA. Injection of Bap subunit resulted in high antibody titers. Decrease in bacterial cell counts of the immunized mice was evident 18 h after challenge. Reaction of antibodies against Bap with several strains suggests that not only immunodominant regions of Bap in *A. baumannii* strains are conserved but also have the same epitope presenting pattern in different strains. Immunodominant region of Bap possesses target sites for a protective humoral immune response to *A. baumannii*. This seems to be a conserved region erecting efficacy of Bap as an appropriate vaccine candidate.

1. Introduction

Acinetobacter baumannii is a nosocomial pathogen and causes severe infections like bacteriemia, pneumonia, meningitis, urinary tract and wound infections [1]. This organism is a particular problem in intensive care units where numerous outbreaks have been extremely difficult to control. The rapid emergence and global dissemination of *A. baumannii* as a major nosocomial pathogen is remarkable and demonstrates its successful adaptation to the 21st century hospital environment [2]. Although a number of studies have attempted to describe the mechanism of virulence of the organism, precise mechanisms involved in the establishment and progression of *A. baumannii* infection are unclear. The organism is not known to produce either diffusible toxins or cytolysins, and a few virulence factors have been identified [2]. Biofilm-dependent production of poly-β-(1-6)-*N*-acetylglucosamine (PNAG) which is known virulence factor in various PNAG producing bacteria, could be an important virulence factor for this emerging pathogen [3]. Interest in *A. baumannii* has intensified owing to its seemingly endless capacity to acquire antimicrobial resistance [4]. This remarkable resistant phenotype could be attributed to the ability of *A. baumannii* clinical strains to form biofilms on abiotic and biotic surfaces [5,6]. Biofilms are structured communities of bacteria encapsulated within a polymeric matrix called exopolysaccharide (EPS) and are of special significance in medicine [7]. There are several conceptual, sequential stages in bacterial biofilm formation [8]: reversible primary attachment of individual cells to a surface, progression to irreversible attachment mediated by exopolysaccharide, early development and maturation of biofilm architecture, and finally dispersal of single cells from the biofilm. In the case of the interaction with abiotic surfaces, genetic and molecular analyses showed that biofilm initiation depends on pilus production via the chaperone-usher pili assembly system [6]. Major outer membrane protein A (OmpA) also proved to be essential for the ability of *A. baumannii* to attach to biotic surfaces [9]. Identifying a staphylococcal biofilm associated protein (Bap) [10] homologue, in a bloodstream isolate of *A. baumannii* was the first identification of a specific cell surface protein directly involved in biofilm formation by *A. baumannii* [5]. It has been suggested that $Bap_{A.\ baumannii}$ is involved in intercellular adhesion within the mature biofilm [5]. Bap-related proteins are present on the bacterial surface; confer upon bacteria the capacity to form a biofilm;

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show a high molecular weight; contain a core domain of tandem repeats; play a relevant role in bacterial infectious processes and can occasionally be contained in mobile elements [5,11]. $Bap_{A.\ baumannii}$ contains 8620 amino acids, making it one of the largest bacterial proteins ever described. It has a predicted pI of ~3, making it one of the most acidic bacterial proteins [5]. Seven tandem repeat modules of Bap constitute the main components of functional and conserved regions [12]. In our previous study we have analyzed the structure of $Bap_{A.\ baumannii}$ with the aim of introducing the conserved functional domains of Bap as appropriate vaccine candidates [12]. One general requirement for any potentially useful vaccine candidate is that its target antigen needs to be widely expressed in human clinical isolates [3,13]. Loehfelm et al. (2008) work showed that biofilm associated protein appears to be common in *A. baumannii* clinical isolates [5]. Surface epitopes of Bap are conserved among approximately 43% *A. baumannii* isolates recovered during the U.S. military health care system outbreak [5]. The virulence of clinical isolates of Acinetobacter spp. has been studied in a mouse model of acute systemic infection induced by intrapretonealy injection [14] and shown to be a good model for *A. baumannii* infection studies. In previous study we demonstrated four regions of *A. baumannii* biofilm associated protein were effective antigens. All regions were predicted to be conserved and functional in native protein [12]. In the present study we put a 371 amino acid subunit of those *in silico* findings into a practical design in order to analyze its reliability and to evaluate its immunogenicity and protectivity on murine model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. baumannii strains isolated from patients in Khatamolanbia hospital (Tehran). *Escherichia coli* BL21 (DE3) cells were used for protein production. Transformants were grown on LB medium containing 50 μg/ml kanamycin. All *A. baumannii* strains were grown in Luria-Bertani (LB) broth or on nutriant agar culture medium at 37 °C.

2.2. Animals husbandry

BALB/c mice, 4–6 weeks old (16–22 g), were procured from the Razi Institute, Tehran, Iran. Mice were housed in clean standard and well-aerated conditions in the animal care facility at Shahed University. Research was conducted in compliance with the Animal Welfare Act and regulations related to experiments involving animals. The principles stated in the Guide for the Care and Use of Laboratory Animals were followed.

2.3. Gene amplification and plasmid construction

A. baumannii strain Kh0060 was used for genome purification. PCR was performed using Bap gene specific primers. The forward primer, was (5′-TTCTAGAATTCATGGCAAATACAGTGGTCACTGTTGTA-3′), corresponding to amino acid positions 706 to 714 of the mature 854-kDa protein (Bap) of *A. baumannii*. The reverse primer, was (5′-TCTATAAGCTTTTATGTCGGATCGTTCACTGTACCA-3′), corresponding to amino acid positions 1054 to 1061. Forward and reverse primers had 5′end *EcoRI* and *HindIII* restriction sites respectively (underlined) for insertion into an expression vector. Restriction mapping was conducted on PCR product with *BclI* and *TaqI* restriction enzymes. The PCR product was digested with *EcoRI* and *HindIII* and inserted into the pET28a(+) vector at the corresponding sites. The new construct was named pET28a(+)–AbBap. Plasmid sequencing was conducted using 23 ABI 3730XLs automatic sequencer at Macrogen Inc. (Seoul, Korea) and was then transformed into *E. coli* BL21 (DE3). The transformants were grown in LB medium containing 50 μg/ml kanamycin. Surviving colonies were picked up for further analyses.

2.4. Expression and purification of recombinant protein

E. coli BL21 (DE3) cells harboring the pET28a(+)–AbBap expression constructs were grown in LB medium supplemented with 50 μg/ml kanamycin at 37 °C with shaking (220 rpm) to an OD_{600} of 0.6, and then induced with 1 mM IPTG. After 4 h of inductionat 37 °C, cells were collected by centrifugation at 5000 rpm for 10 min. The cell pellet was resuspended in buffer B (denaturating lysis/binding buffer). The lysate was then sonicated (6 times, 10 s at 200 w with a 10 s cooling period between each burst) using a sonicator equipped with a microtip. The lysate was then centrifuged at 14000 rpm for 20 min at 4 °C to pellet the cellular debris. The supernatant was applied to an Ni–NTA agarose affinity column. The column was washed stepwise with buffer C (denaturing wash buffer, pH = 6.3) and buffer D (denaturing elution buffer, pH = 5.9). Buffer E (denaturing elution buffer, pH = 4.5) was used to elute Bap recombinant subunit protein. The buffer solutions contained 8 M urea. The protein was analyzed by SDS-PAGE 10%. Sequential dialysis was carried out against PBS (pH 7.4) containing 6, 4, 2 and 0 M urea respectively. Concentration of purified protein was determined according to Lowry et al. [15] method with bovine serum albumin (BSA) as standard.

2.5. Dot immunoblotting

In order to confirm the expression of Bap, dot immunublotting was performed employing anti-His. 0.5 μg of each protein sample from induced transformed cell lysates and purified recombinant Bap subunit were adsorbed and dried onto a nitrocellulose membrane strip. The uninduced transformed cell lysates served as control. The strip was incubated in the blocking buffer of 3% BSA, with gentle shaking for 1 h at room temperature. The strip was then washed 3 times with PBST before incubation with the diluted anti-His conjugated with horseradish peroxidase (1:8000) for 1 h. The strip was washed 3 times for 5 min in PBST. The membrane was visualized with diaminobenzidine substrate until brownish dots were observed. Washing with PBST terminated colour development.

2.6. Immunization of mice

Thirty mice received three vaccinations of 10 μg of the recombinant Bap protein at 2 week intervals. The initial vaccinations were emulsified with complete Freund's adjuvant (Sigma), and the next two with incomplete Freund's adjuvant (Sigma). Blood samples were collected 10 days post-injection through infra-orbital plexus. An additional 30 BALB/c mice that received PBS and Freund's adjuvant, served as a control group.

2.7. ELISA with recombinant Bap subunit

Recombinant Bap subunit was first diluted with coating solution to an optimal concentration (20 μg/ml) in order to coat a 96-well plate with. The resulting solution was then added into each well (100 μl per well) and incubated for 12–18 h at 4 °C. To block the unoccupied sites, wells were washed once with PBS plus 0.05% Tween 20 (PBST), and then incubated with 100 μl of PBST plus 5% skimmed milk for 1 h at 37 °C. After washing the plates 3 times with PBST (100 μl per well), serial dilutions of each serum ranging from 1:800 to 1:102 400 were added to the wells in triplicate and incubated at 37 °C for 1 h. Plates were washed 3 times, again as described above. 100 μl per well of Horseradish peroxidase-

conjugated (HRP-conjugated) secondary antibody (diluted 1:1000 in PBST) was added and the plates incubated for 1 h. Plates were then washed 3 times with PBST and were then incubated with 100 μl per well of 3,3′,5,5′-tetramethylbenzidine solution (TMB) as substrate until a desired absorbance was reached. The reactions were stopped by the addition of 2 M H_2SO_4. The optical density of the samples was measured at 450 nm using an ELISA plate reader.

2.8. Whole cell ELISA

Clinical strains and Bap negative strain of *A. baumannii* were cultured overnight in LB broth. Cells were harvested and resuspended in PBS and diluted to an A_{550} of 0.3. 50 μl of this suspension was added to each well of 96-well plates. The plates were dried at 37 °C. After blocking with 200 μl of PBS containing 5% skimmed milk for 1 h, the plates were washed five times with PBS and incubated with immunized mice sera diluted in PBS for 1 h. The plates were then washed and incubated with HRP conjugated secondary antibody for 1 h. Plates were washed 5 times with PBS and then incubated with 100 μl per well of TMB as substrate until a desired absorbance was reached. The reactions were stopped by the addition of 2 M H_2SO_4. The optical density at 450 nm was read on ELISA plate reader. The preceding procedure was followed for unimmunized mice sera with *A. baumannii* and immunized sera with a Bap negative strain keeping other conditions constant.

2.9. Determination of lethal dose (LD_{50})

The 50% lethal dose (LD_{50}) was determined in the following manner: *A. baumannii* at doses ranging from 10^4 to 10^9 cfu were administered intraperitoneally to six groups of five BALB/c mice per group. LD_{50} was estimated by the Probit method [16] from the number of survivals on day 5.

2.10. Challenge of immunized mice

Seven days after the final booster dose, the mice were challenged intraperitoneally with *A. baumannii* Kh0060. Survival was monitored until day 40. Fresh inocula were prepared for each experiment from a frozen stock of *A. baumannii*. Six groups of five mice in each were injected with 10^8 to 10^{13} cfu of *A. baumannii* in 100 μl phosphate-buffered saline. Control group was exposed to the same bacterial treatment.

2.11. Bacteriological and histopathological examination

For quantitative bacteriological studies, 7 days after the last immunization, 10^9 cfu of *A. baumannii* Kh0060 were injected intrapritoneally to immunized and unimmunized groups. Liver and spleen samples were aseptically collected at 6, 12, 18, 24, 48, 72 and 96 h. The samples were weighed and homogenized in 1 ml of PBS. Serial 10-fold dilutions of the homogenates were plated onto Nutrient Agar. For histopathological examination, the tissue samples were fixed in formalin. Paraffin embedded tissues were cut into 6-μm-thick sections. The sections were stained for light microscopy with hematoxylin and eosin (HE).

2.12. Statistical analysis

All data are expressed as mean ± standard deviation (SD). One-way ANOVA with post hoc Scheffé tests determined statistical significance of ELISA and whole cell ELISA results. *P*-values were calculated by Student's *t* test to determine the significance of differences in cell counts among the experimental groups. Chi-square analysis was used to determine if number of viable and dead mice varied significantly. SPSS 16.0 was used for data analysis. *P* values of <0.05 were considered as significant.

3. Results

3.1. PCR amplification

The size of PCR amplicon of the fragment encoding one conserved region of Bap was 1113 bp. The fragment of appropriate size and digestion patterns of amplified Bap subunit with *BclI* and *TaqI* restriction enzymes were observed on 1% agarose gel.

3.2. Expression and purification of Bap subunit

SDS-PAGE 10% with Coomassie staining confirmed high protein purity. The band corresponding to desired molecular mass of approximately 41 kDa appeared differently. The electrophoretic mobility of the recombinant protein roughly corresponds to its molecular mass, while migration of this protein would be in accordance with the expected molecular mass of protein. This protein displays an anomalous electrophoretic behavior on standard SDS-PAGE, revealing that the protein did not bind SDS under the SDS-PAGE conditions [17].

3.3. Dot immunoblotting

Electrotransference of samples from SDS gels to Western membranes was failed even after 2 h of transference. Anti-His was able to recognize the protein clearly upon dot blotting, suggesting a failure in the transferring process in Western blotting. The reaction of anti-His revealed greater color intensities on induced transformed cell lysate and purified Bap subunit compared to the control.

3.4. Humoral responses to Bap

Mice immunized with purified Bap subunit protein showed significant rise of IgG antibodies. The antibody titer increased significantly ($P < 0.001$) after the second booster, whereas animals received adjuvant and PBS, as control had no Bap-specific antibodies in serum (Fig. 1).

3.5. Whole cell ELISA

Antibody against Bap subunit reacted with most experimental strains. Significant OD values were detected when antibody was exposed to *A. baumannii* Kh0060, and other strains compared to controls (Fig. 1).

3.6. Challenge studies in mice

The LD_{50} of *A. baumannii* was determined at 10^7 bacteria per mouse. Most of the immunized animals were protected from a lethal dose of *A. baumannii*. Whereas none of unimmunized mice survived within 24 h of inoculation (Fig. 2).

3.7. Enumeration of A. baumannii in liver and spleen

Bacterial load of *A. baumannii* in liver and spleen was approximately at an equal level. Unimmunized mice could not be examined after 18 h because of their death as a result of the established infection. Decrease in bacterial cell counts of the immunized mice was evident 18 h after challenge. No microorganism was recovered from the tissue samples of immunized groups after 96 h (Fig. 3).

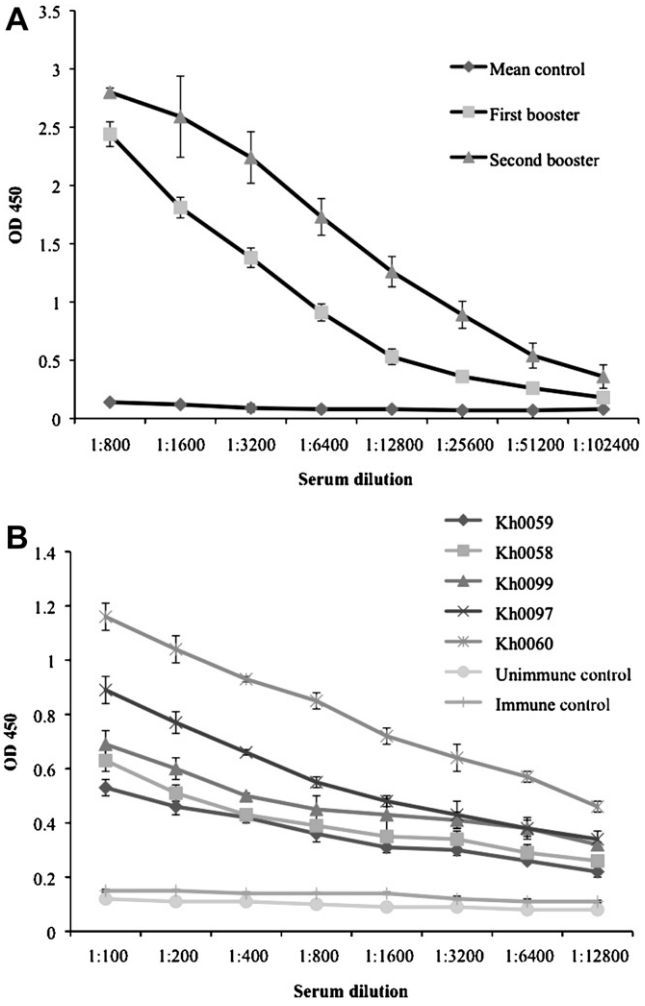

Fig. 1. Indirect ELISA of serum with Bap subunit protein (A); The sera were collected after first and second immunization and assessed for specific IgG. Antibody titers increased significantly ($P < 0.001$) in the second booster. Non immunized mice sera were used as control. Whole cell ELISA results (B); Antibodies exposed to five strains of *A. baumannii*. Significant ($P < 0.05$) OD values were observed in all experimental strains as compared to control groups. Unimmunized control: unimmunized mice sera with *A. baumannii*. Immunized control: immunized sera with a Bap negative strain of *A. baumannii*.

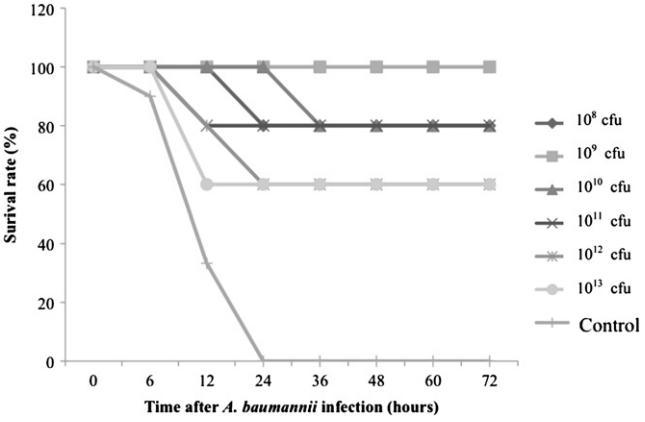

Fig. 2. Survival rate analysis of mice after challenge. Chi square analysis with a 95% confidence interval showed that the immunized mice were significantly protected compared to the control.

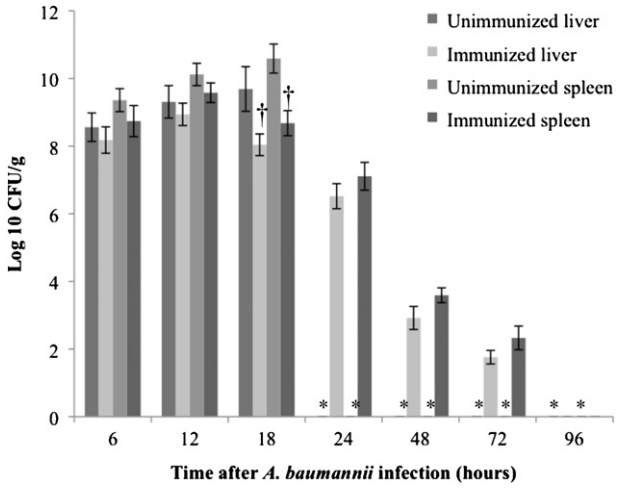

Fig. 3. Viable bacterial cell counts in the liver and spleen of immunized and unimmunized mice infected intraperitonealy with *A. baumannii*. None of unimmunized mice infected with *A. baumannii* survived after 24 h as presented in figure (*). †$P < 0.001$ compared with unimmunized mice at same interval.

3.8. Histopathology

The histopathological study showed the appearance and general composition of the lymphocytes developed in each group (Fig. 4). All the lesions were incipient because animals were sacrificed 1 day

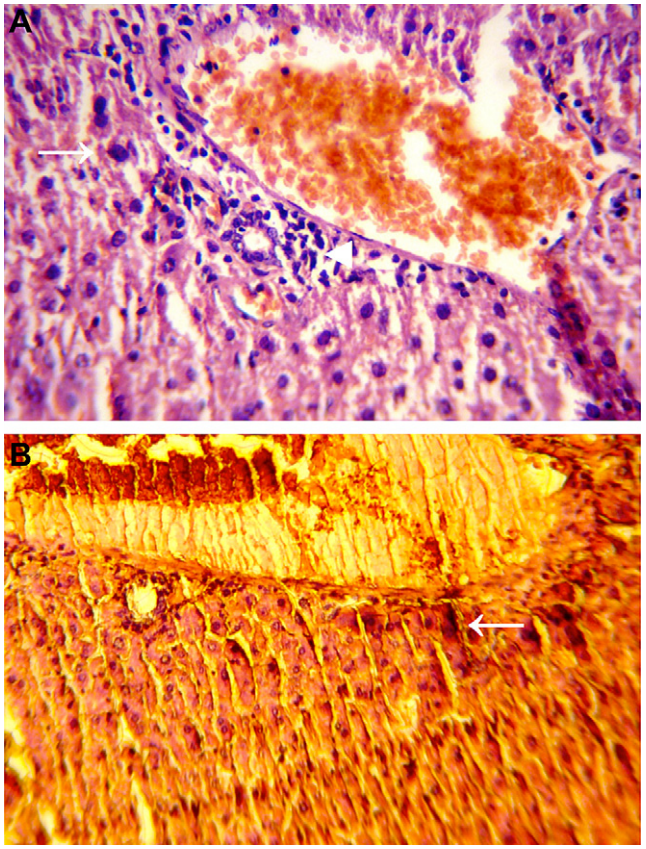

Fig. 4. Micrograph (× 400) of liver tissue of unimmunized (A) and immunized (B) mice after *A. baumannii* challenge, stained with Haematoxylene and Eosin (HE). Moderate inflammation is observed in liver tissue of unimmunized mice and slight inflammation in tissue of immunized group. Lymphocyte aggregation (arrowhead) and apoptosis (arrows) are evident in samples.

post-challenge, which is considered an early stage in the development of the disease. Portal tract inflammations, mainly constituted by lymphocytes, were more evident in unimmunized animals inoculated with 10^9 cfu. Slight inflammation and aggregation of lymphocytes characterized the liver samples from immunized group (Fig. 4). Unimmunized mice livers and spleens showed increased volume as observed by naked eyes.

4. Discussion

The recent global expansion of multi- or pan- drug resistant clones of *A. baumannii* has often resulted in situations where there are very few effective antibiotics [2]. The design, selection and production of recombinant subunit vaccines [18], might be one option for encountering the growing problem of *A. baumannii* infections. More than 20 antigenic determinants and 55 discontinuous B-Cell epitopes have been predicted for Bap subunits [12]. Theoretical isoelectric point for this Bap subunit was estimated as 3.4, at http://expasy.ch/cgi-bin/pi_tool [19], low electrophoretic mobility of Bap subunit in SDS-PAGE experiment may be explained by this remarkable low pI, since very acidic proteins do not bind SDS under standard SDS-PAGE conditions [17]. In the present study it was found that Bap was highly immunogenic and resulted in high antibody titers (Fig. 1). Two important factors, repetition and hydrophilicity, may explain the antigenicity of the Bap recombinant subunit [12]. Significant survival rate improvement was observed in challenged mice 7 days after the last immunization (Fig. 2). This improvement was more evident on day 4 onwards where bacterial clearance took place (Fig. 3). Reaction of antibodies against Bap with several strains (Fig. 1) suggests that not only immunodominant regions of Bap in *A. baumannii* strains are conserved but also possess the same epitope presenting pattern in different strains. Homologous proteins from different species may have a high degree of sequence identity but have markedly different epitope presentations [20]. It may be proposed that antibodies against this subunit interfere with adhesion and accumulation of *A. baumannii* depriving bacterium of virulence and holding it in a planktonic phase. Bap involves in early development and maturation of biofilm architecture [5]. Since steps of biofilm formation occur particularly after exopolysaccharide secretion [8], possibility of surrounding by PNAG of Bap surface epitopes is not ruled out. *Staphylococcus epidermidis* reported to possess PNAG [21], the biofilm extra matrix in this case, did not pose an overall diffusion barrier to the antibody [22]. This supports the notion that antibodies are able to penetrate *A. baumannii* biofilms. Our results suggest that access to the bacterial cell surface through PNAG by antibody is sufficient to impart bactericidal effect (Fig. 4). Specific and nonspecific adsorption of antibodies to Bap might inhibit adhesion and play a critical role in the prevention of development and maturation of biofilm. This inhibition could simply be explained by conformational changes in native Bap while there is sufficient evidence indicating that antigens and antibodies can significantly change their molecular conformation upon binding [23,24]. The findings indicate that the selected subunit protein is produced in the biofilm and is present on the cellular envelope.

5. Conclusions

Immunodominant region of Bap possesses target sites for a protective humoral immune response to *A. baumannii*. This seems to be a conserved region erecting efficacy of Bap as an appropriate vaccine candidate. Immunogenic properties are implemented by the major conserved functional regions of this molecule.

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