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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-