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The role of filamentous hemagglutinin adhesin in adherence and biofilm formation in *Acinetobacter baumannii* ATCC19606^T

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ABSTRACT

Filamentous hemagglutinin adhesins (FHA) are key factors for bacterial attachment and subsequent cell accumulation on substrates. Here an FHA-like Outer membrane (OM) adhesin of *Acinetobacter baumannii* ATCC19606^T was displayed on *Escherichia coli*. The candidate autotransporter (AT) genes were identified in *A. baumannii* ATCC19606^T genome. The exoprotein (FhaB1) and transporter (FhaC1) were produced independently within the same cell (FhaB1C1). The *fha*C1 was mutated. In vitro adherence to epithelial cells of the recombinant FhaB1C1 and the mutant strains were compared with *A. baumanni* ATCC19606^T. A bivalent chimeric protein (K) composed of immunologically important portions of *fha*B1 (B) and *fha*C1 (C) was constructed. The mice vaccinated with chimeric protein were challenged with *A. baumannii* ATCC19606^T and FhaB1C1 producing recombinant *E. coli*. Mutations in the *fha*C1 resulted in the absence of FhaB1 in the OM. Expression of FhaB1C1 enhanced the adherence of recombinant bacteria to A546 bronchial cell line. The results revealed association of FhaB1 with bacterial adhesion and biofilm formation. Immunization with a combination of recombinant B and K proteins proved protective against *A. baumanni* ATCC19606^T. The findings may be applied in active and passive immunization strategies against *A. baumannii*.

1. Introduction

Acinetobacter baumannii is harboring a number of virulence factors. These factors include the ability to obtain essential nutrients such as iron, the adhesion to and subsequent destruction of epithelial cells, and the ability in some strains to produce gelatinase and proteinase that damage host tissues. *A. baumannii* also has the ability to form biofilm, which may play a role in the process of colonization [1]. Biofilm helps the bacteria resist disinfection while allowing the participating cells to acquire resistance genes [2] further facilitating the persistence of the pathogen [3]. Nowadays, scientists have focused on surface antigens [4–8]. Serological methods and fatty acid analysis indicated immunogenicity of lipopolysaccharide purified from *A. baumannii* [9]. Siderophores are secreted in the external melieu where they compete with host iron binding proteins to capture iron by forming iron-siderophore complex. This gets internalized through the specific outer membrane protein receptors, termed as Iron Regulated Outer Membrane Proteins (IROMPs) [10]. Vaccination with outer membrane proteins (OMPs) and antibody-based therapies may be valuable approaches to prevent the morbidity and mortality caused by this pathogen [5,11]. The rOmpA vaccine has been shown to protect mice from lethal infection caused by extreme-drug-resistant (XDR) *A. baumannii*. The rOmpA vaccine resulted in an enhanced Type 2 immune response, accompanied by substantial IL-4-inducing T cell epitope spreading and restricted IFN-γ –inducing epitopes [12]. The refinement of genomic and proteomic techniques are significant prospects for the development of recombinant vaccines. The investigators showed that killed but metabolically active (KBMA) vaccines stimulate strong immune responses [13]. In order to identify a candidate antigen in *A. baumannii*, the immunodominant targets from *A. baumannii* membrane protein preparations were determined following systemic infection [14].

Further to these studies new approaches are needed to expand our understanding of the basic features of this organism essential to control the spread of *A. baumannii* infections which will ultimately end up at development of effective means to prevent and/or treat this harmful pathogen. To gain greater insight into *A. baumannii* virulence factors, our search of *A. baumannii* ATCC 19606^T genome revealed three open reading frames (ORFs) encoding putative protein autotransporter of Two-partner secretion system (TPS) types.

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As implied by the name, there are two major players in TPS systems, the secreted proteins collectively called TpsA proteins and their outer membrane transporter partners collectively called TpsB proteins. The defining feature of the TpsA proteins is the presence of a conserved, approximately 250-residue-long "TPS' domain located at the N-terminus of the mature protein. Most TpsA proteins are large and predicted to form extended solenoid structures, much like classical, type Va AT proteins. TpsB partners are 60 kDa proteins embedded in the outer membrane. The structure of the TpsB transporter FhaC serves as a model for the TpsB family. TpsB proteins are composed of two large moieties. The periplasmic moiety is formed by two successive (polypeptide transport-associated (POTRA) domains involved in the recognition of the TpsA cargo. The C-terminal moiety is embedded in the outer membrane and forms a 16-stranded, anti-parallel β-barrel that delimits a channel thought to be the translocation pore for the TpsA partner [15].

Filamentous hemagglutinin (FHA) is a major virulence factor of *Bordetella pertussis*, the causative agent of the highly transmissible and infectious disease pertussis. This array of adherence factors likely allows the organism to efficiently colonize several different areas of the human respiratory tract. Filamentous hemagglutinin adhesins are key factors for bacterial attachment and subsequent cell accumulation on substrates [16] representing attractive targets in vaccinology. Adhesins have proven to be effective vaccine antigens. For instance, all vaccines currently licensed for use in the United States against *B. pertussis* target the FHA [17]. In this study, we report the identification and characterization of an FHA-like adhesin expressed in the OM of *A. baumannii* ATCC19606^T. Arrangement of the genes encoding TPS are *fha*B1 and *fha*C1 in one ORF and *fha*B2 and *fha*C2 in the other. The present study deals with the role of conserved regions of *fha*B and *fha*C focused on *fha*B1 and *fha*C1system. In silico structural analysis of the protein was carried out. Role of the protein in biofilm formation and adherence to human bronchial epithelial cells were investigated in vitro.

2. Materials and methods

2.1. Bacterial strains, plasmids, cell lines and growth conditions

pET22b and pET28a were from Novagen (USA). These plasmid were procured from biotechnology laboratory of Shahed University, Tehran-Iran. pBAD33 was kindly donated by Prof. Harris D. Bernstein, National Institute of Health, Bethesda. The human bronchial epithelial A549 cells and HeLa cells were purchased as a complete culture system from Iranian Biological Resource Centre and were propagated as per the manufacturer's instructions. The cells were cultured in DMEM medium supplemented with 2 mM L-glutamine, 50 mg ml^{-1} streptomycin, 1 mg ml^{-1} sodium penicillin G and 10% heat-inactivated fetal Bovine serum (FBS) all from Gibco (Invitrogen, Breda, Netherlands) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. *A. baumannii* ATCC 19606^T *and Escherichia coli* BL21 (DE3) from our lab were cultured in Luria–Bertani (LB) broth or on LB agar plates at 37 °C. The recombinant *E. coli* were cultured in medium containing 20 μg/ml Chloramphenicol or Kanamycin,70 μg/ml; Ampicillin100 μg/ml as and where indicated.

2.2. Bioinformatic analyses

A. baumannii ATCC19606^T was searched for highly conserved autotransporter domains using the Pfam database including the autotransporter beta domain (http://pfam.sanger.ac.uk//family/PF03797) and the hemagglutination activity domain (http://pfam.sanger.ac.uk//family/PF05860). The resulting sequences containing these domains were used to identify additional autotransporters in *A. baumannii* genome available in un-annotated form via the Sanger Institute (http://www.sanger.ac.uk/Projects/), which was facilitated by investigating the available sequence using the National Microbial Pathogen Database Resource (NMPDR).

A number of parallel Bioinformatic approaches were used to identify the candidate autotransporter (AT) genes in *A. baumannii* based on the available sequences in NCBI. In this work a large ORF homologous to *fha*B and *fha*C of *B. pertussis* were named *fha*B1 and *fha*C1respectively. The signal peptide and the secondary structural features of *fha*B1 and *fha*C1 were predicted using the SignalP3.0 program, available at (http://www.cbs.dtu.dk/services/SignalP/) and the PSIPRED software respectively. SignalP (http://www.cbs.dtu.dk/services/SignalP/) was used to identify potential signal peptide encoding regions of the predicted AT coding sequences. BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Pfam domain searches of each putative AT peptide were used to define the conserved beta barrel transport domain in NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and Pfam data-bases. A model of the three-dimensional (3D) structure of FHA residues was built by the combined use of phyr2 and I-TASSER. The *A. baumannii* homologues of the filamentous hemagglutinin genes were identified by BLAST searches of the *A. baumannii* genome European Nucleotide Archive database.

2.3. Amplification and cloning of *fhaB1* and *fhaC1*

A. baumannii genomic DNA was isolated using the Invitrogen Easy DNA kit under the conditions recommended by the manufacturer. Plasmid DNA was obtained with the Vivandis Kit according to the manufacturer's specifications. Unless otherwise stated all PCR experiments were performed using Maxime PCR PreMix Kit (i-pfu) (Interon). *fha*B1 open reading frame was amplified with the oligonucleotide primers P1 (CTTACATATGATGAACAAGAATAG TTATCGCATTATTT)/P2(CTTA CT CG AGTCAATTTTTTTTTCTTTTCTCA AGA). Amplicons of *fha*C1 was generated with primers P5 (GTAAC ATATGATGCAAAACAAAATTTTTTTTACTC)/P6 (GT AA GTCGACTTAAT AAAAAGCATTTAGACTAAATCC). The PCR fragments specifying *fha*B1 and *fha*C1 were cloned, yielding the plasmids pET22-B1 and pBAD33-C1 respectively.

2.4. Isogenic mutations in the *fhaC1*

FhaC1 sequence was disrupted by insertion of Kanamycin gene using EZ-Tn5 < oriV/KAN-2 > insertion kit (Epicenter). Both pET22-B1 and the mutated plasmid named pBAD33-mC1 were introduced into *E. coli* BL21(DE3) cells. The transformed cells were named mFhaB1C1. Chloramphenicol resistant (CmR, specified by the vector pBAD33) and Kan-resistant (KanR, specified by the EZ-TN5 < oriV/KAN-2 > insertion kit) colonies were selected and the plasmids were analyzed by PCR using primers P5 and P11 (GAGCC AATATGC GAGAACACCCGAGAA) for the first PCR and P6 and P12 (GCCAACGACTACGCACTAGCCAAC) for the second PCR. Proper integration of Kan into FhaC1 was verified by amplifying the FhaC1 loci by PCR and analyzing the PCR product by restriction digest.

2.5. Expression and purification of the recombinant proteins

In order to obtain polypeptides for antibody production three recombinant proteins were produced using synthetic genes. These genes were exoprotein named as "protein B", The channel protein named as "protein C" and a chimeric protein called "K". The exoprotein "B" was composed of immunologically important conserved regions of epitopes *fha*B1 and *fha*B2. The channel protein "C" was composed of immunologically important conserved regions of epitopes *fha*C1 and *fha*C2.

A bivalent recombinant chimeric protein "K" was composed of proteins B and C. For high-level expression, the *K* gene was synthesized with codon bias of *E. coli*. These genes were used to generate bivalent proteins by linkers (EAAAK). For more flexibility and efficient separation [18] four repeated sequences of EAAAK were introduced between the *B* and *C* genes. The gene encoding target protein verified by Gen-Script (NJ, USA) was synthesized by Pars Biomatic (Iran) and delivered to us in pUC57 cloning vector.

The gene was subcloned into pET28a and the construct was called "pET-K". A similar strategy was used in the construction of the plasmids "pET-B" and "pET-C". The recombinant proteins individually expressed in *E. coli* BL21(DE3) were separated from inclusion bodies by suspending the cells in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea) followed by sonication. The proteins were purified under denaturing conditions using the His-Bind resin system (Novagen). Urea was removed from these preparations by dialyzing at 4 °C while gradually decreasing its concentration (6 M > 4 M > 2 M > 0 M) over a period of 8 h.

2.6. Production of polyclonal antibodies

The immunizations were conducted in mice with purified B, C and K proteins. 20 μg from each of the purified recombinant proteins was subcutaneously injected to each of five male Balb/C mice (20–25 g) groups on days 0, 12, 24 and 36. Each protein was mixed with equal amount of Freund's adjuvant to a final volume of 0.2 ml. The complete adjuvant was used at the first injection and the incomplete one for the subsequent injections. PBS mixed with the adjuvant served as a negative control. Blood samples were collected 14 days after the third and fourth injections.

2.7. Sera titration

ELISA was employed to titrate each of anti-B, anti-C and anti-K antibodies in sera of the vaccinated mice. Polystyrene 96-well plates were coated with 20 μg from each purified proteins in 100 μl coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.8) and was then incubated overnight at 4 °C. The plates were washed with PBS/T and blocked for 45 min at 37 °C with 5% skimmed milk in PBS/T. The plates were incubated with serial dilutions of each B, C and K sera at 37 °C for 120 min followed by three to five washes. The plates were incubated at 37 °C for 60 min with goat anti-mouse immunoglobulin G horseradish peroxidase (IgG-HRP, 1:100,000). The wells were then added with 100 μl of TMB for 15 min at room temperature. The reaction was stopped with 100 μl of 2 M $H_2SO_4$and the absorbance was read at 450 nm.

2.8. Expression of FhaB1 and FhaC1 proteins

In order to obtain evidence that FhaB1 and FhaC1 function as TPS, the two proteins were produced independently within the same cell. pET22b-B1 and pBAD33-C1 were transformed into BL21(DE3) for the induction of FhaB1 under the control of an IPTG-inducible promoter, and pBAD33-C1 encoding FhaC1with a promoter under the control of arabinose. This cell encoding both FhaB1 and FhaC1 was named as FhaB1C1. The cultures were incubated with one or both inducers for 3 h and the cells were harvested by centrifugation. The cell pellets and culture supernatants were then probed by Western blotting and whole bacterial cell ELISA with anti-B antisera.

2.9. Localization of FhaB1 and FhaC1 on OM

Logarithmic growth ($OD_{600} = 0.5$) of FhaB1C1 harboring bacteria was used in the experiments. The plates were coated overnight at 4 °C with 100 μl of bacterial suspension per well. After three washes with PBS containing 0.05% (w/v) Tween 20 (PBS-T), the remaining binding sites on the wells were blocked with PBS containing 5% (w/v) skimmed milk (30 min). Wells were added with 100 μl of anti-B serum and incubated at 37 °C for 120 min. After three washing steps, 100 μl of goat anti-mouse IgG peroxidase conjugate (Sigma), diluted to 1:8000 in PBS-T, were added (37 °C, 1 h). After three more washes, 100 μl per well of substrate, consisting of TMB (3,3′,5,5′-tetramethylbenzidine) was added and the reaction was stopped by the addition of 100 μl per well of 2 M sulfuric acid. Absorbance at 450 nm was determined with a plate reader.

2.10. Adherence/invasion assay

Adherence/invasion of the recombinant (FhaB1C1) and the mutant (mFhaB1C1) strains were compared with *A. baumannii* and *E. coli* BL21(DE3) using A549 and HeLa monolayer cells in vitro [17,19]. Briefly, 2.5×10^5 human A549 and HeLa cells were seeded into each well of a 6-well tissue culture plate and incubated overnight before use. The bacterial strains to be tested were also streaked onto appropriate agar plates and grown overnight followed by inoculation of the cells into 50 ml of fresh medium at an OD_{620} of 0.02. The expression was induced by the addition of 100 μM isopropyl-D-thiogalactopyranoside (IPTG) and 0.2% arabinose at OD_{620} of 0.5. The bacterial cells were harvested after 3 h by centrifugation at 3700 × *g* for 10 min at 4 °C and re-suspended into sterile culture medium without antibiotics, and inoculated at 10^6 CFU per well in triplicate onto monolayers of the 10^5 cells per well human cells. The tissue culture plate was then incubated for 3 h at 37 °C. This assay was carried out for both recombinant *E. coli* and *A. baumannii*. Non-adherent bacteria were removed by gently rinsing the wells five times with phosphate-buffered saline supplemented with 0.15% (W/V) gelatin (PBSG), and the human cells were released from the plastic support of the tissue culture plate by adding 100 μl of a solution containing 0.05% trypsin and 0.02% EDTA. The released human cells were suspended and serially diluted in 500 μl of PBSG buffer. The dilutions were spread onto LB agar plates containing antibiotics to determine the number of viable bacterial cells attached to human cells. Adherence is defined as the percentage (standard deviation) of bacteria attached to the human cells compared with the initial inocula. The assay was repeated at least three times.

2.11. Biofilm formation assay

The ability of *A. baumannii*, *E. coli* BL21(DE3), mutated and non mutated recombinant *E. coli* to form a biofilm on an abiotic surface was quantified as described by Brossard et al. [20]. Adhesion of bacteria to 96-well polycarbonate microplate surfaces was carried out by inoculating 20 μl of bacteria grown overnight in LB into 180 μl of M9 biofilm medium. The plates were incubated for 5 days at 30 °C and the biofilm formation was estimated by crystal violet staining method. The wells were gently washed 3 times with 200 μl of phosphate-buffered saline (PBS), dried and stained with 1% crystal violet for 15 min. The wells were then rinsed, and the crystal violet was solubilized in 100 μl of ethanol-acetone (80:20, v/v). The OD_{595} was determined using a microplate reader. Each assay was performed in triplicate. The following values were assigned for biofilm determination: $OD_{595} < 1$ as non-biofilm forming and $1 < OD_{595} < 2$ as biofilm-forming. In order to plot a graph that could be compared with percent hydrophobicity, OD_{595} readings of biofilm formation test were multiplied by 100.

2.12. Hydrophobicity assays

Cell hydrophobicity was determined using a standard microbial adhesion to hydrocarbon (MATH) test [21,22]. Briefly, bacterial cells

were cultured in LB broth at 37 °C in a shaking incubator. The cells were collected by centrifugation, washed twice, resuspended with PUM buffer (22.2 g/l $K_2HPO4 \cdot 3H_2O$, 7.26 g/l KH_2PO_4, 1.8 g/l urea, 0.2 g/l, $MgSO_4 \cdot 7H_2O$; pH 7.1) and adjusted to an OD_{600} of 1followed by addition of 160 S μl of p-xylene and vortexed for 120 s. The suspensions were allowed to stand stationary for 15 min. Hydrophobicity of the bacterial cells was determined by their adhesion to p-xylene. The percent hydrophobicity was calculated as $[1-(OD_2-OD_1)] \times 100$ where OD_2 is the optical density after addition of p-xylene. All assays were conducted in duplicate at least twice using fresh samples each time.

2.13. Hemagglutination assay

Hemagglutinating activity of *A. baumannii* and recombinant *E. coli* was determined by micro-hemagglutination test using 96-well round-bottom plates and fresh human group O and AB, Rh positive erythrocytes [23]. Bacteria grown on LB plates at 37 °C for 24 h were suspended and serially diluted in PBS. The starting concentration was 10^{10} CFU/ml. A suspension of 1% erythrocytes was added to each well and mixed. Wells containing only the suspension of erythrocytes served as negative control. A small pellet of erythrocytes at the well bottom after 1 h incubation at 4 °C were considered negative as against positive reactions exhibiting an even sheet of erythrocytes across the wells.

2.14. Hemolytic assay

In order to evaluate the synthesis of the TPS exoprotein and its cognate transporter, hemolytic activity in recombinant *E. coli* was assayed. The recombinant *E. coli* FhaB1C1 was streaked on blood agar containing 5% sheep blood, 100 mM IPTG and 0.2% Arabinose.

2.15. Challenge study

Minimum Lethal Dose (MLD) of *A. baumannii* was determined as 10^8 CFU/mouse. Two weeks after the last booster, the vaccinated mice were injected intraperitoneally with *A. baumannii* ATCC 19606^T at 10^9 and 10^{10} CFU concentrations. The challenged animals were monitored for 7days and the number of deaths for each group was recorded. All procedures and ethical issues were followed in compliance with relevant laws and institutional guidelines of Shahed University.

10^{10} CFU of recombinant *E. coli* induced to produce FhaB1C1-were administered intraperitoneally (i.p) to each of the 5 week old immunized and unimmunized Balb/C mice. Mortality was scored for a 7 day period.

2.16. Statistical analysis

Initially, the data were tested for normality by SPSS version 22 and were then analyzed for the traits using analysis of variance and Duncan's multiple range test ($P < 0.05$). The Graph pad prism version No 6 was used for plotting the graphs.

3. Results

3.1. Bioinformatic analysis

Analysis of the genomic sequence of *A. baumannii* using tblastn (NCBI) identified ORF (1740 bp) with 25% similarity to the *fha*C gene product of *B. pertussis* Tomaha1(NP-880575.1, GenBank). The *fha*C1 gene sequence analysis predicted a 58-kDa protein of 579 aa. Using SignalP3.0, a putative signal sequence cleavage site was detected between residues 23and 24 (AYA▼ID). Further analysis with PSIPRED revealed that the ORF potentially contains 18 to 19-strands, suggesting a porin conformation. Database searches using Pfam also demonstrated that the N-terminus of the gene product possesses a POTRA2 domain (PF08479), which is present in a number of porin-like proteins responsible for the transport of polypeptides across the OM of gram-negative bacteria. The ORF was named *fha*C for *A. baumannii* FhaC1 protein. Sequence analysis of upstream of *fha*C revealed two ORFs of 5.2 and 3.2 kb identified in the *A. baumannii* genome deposited through European Nucleotide Archive (ENA). The deduced amino acid sequences corresponding to these large ORFs were used as queries for BLAST searches (NCBI), which revealed limited overall sequence similarity to other bacterial TpsA proteins.

A. baumannii FhaB1 protein was so named for their similarities to *B. pertussis* FhaB and other TPS exoproteins. *A. baumannii* FhaB1 had an estimated molecular mass of 110 kDa (1072 aa). Two hundred amino acids were identical in FhaB1 constituting 48% of the total TPS pathway. Further analysis of the TPS domains of FhaB1 revealed an N-terminal extension linked to the signal peptide. A signal sequence cleavage site was detected between aa 26 and 27 (AVA▼EN).

A potential hemagglutination activity domain reported in the Protein Families Database (PF05860) between aa 80 to 217, was found in the N-terminal portions of the amino acid sequences of FhaB1. This region corresponds to 1–130 residues of FhaB of *B. pertussis*, as the TPS domain of TpsA proteins.

3.2. Expression, purification and immunogenic property of the chimeric proteins

The synthetic genes of exoprotein (B), the channel (C) and the chimeric (K) proteins were expressed in *E. coli* (BL21DE3) with 6X-His-tag.SDS-PAGE analysis revealed the presence of 56 kDa, 31 kDa and 29 kDa bands pertaining to K, B and C respectively (Fig. 1a). The recombinant proteins were then confirmed by Western blotting using anti His-tag antibody (Fig. 1b).

Mice immunized with B, C and K purified proteins showed significant rise of antibodies ($P < 0.01$) as compared to the control (Fig. 2).

3.3. Expression and localization of fhaB1 and fhaC1

*fha*B1 (3200 bp) and *fha*C1 (1740 bp) were cloned and transformed into *E. coli* BL21(DE3). The prominent 110 kDa band was detected by anti-mouse antisera generated against B epitopes (Fig. 3). These proteins were not seen in the mutant strain. Whole cell ELISA analyses exhibited the presence of FhaB1 in the recombinant bacterial OM (Fig. 4) whereas mFhaB1C1 did not reveal the protein in the OM fraction using anti-B.

3.4. Adherence assay

The possibility that FhaB1 functions as an adhesin was also tested by monitoring bacterial adherence to A549 and HeLa cells using a standard adherence assay. Fig. 5 shows 20 and 28 percent adherence of the mFhaB1C1 to HeLa and A549 cells respectively as compared to 55 and 63 percent adherence of FhaB1C1 to HeLa and A549 cells respectively. Adherence of *A. baumannii* and *E. coli* BL21(DE3) to HeLa cells were 15 and 26% while those of the same bacteria to A549 cells were 26 and 30 percent respectively.

3.5. Biofilm formation and hydrophobicity assay

The biofilm formation by FhaB1C1 was determined (Fig. 6). Quantitative analysis of biofilm formed on polystyrene surfaces

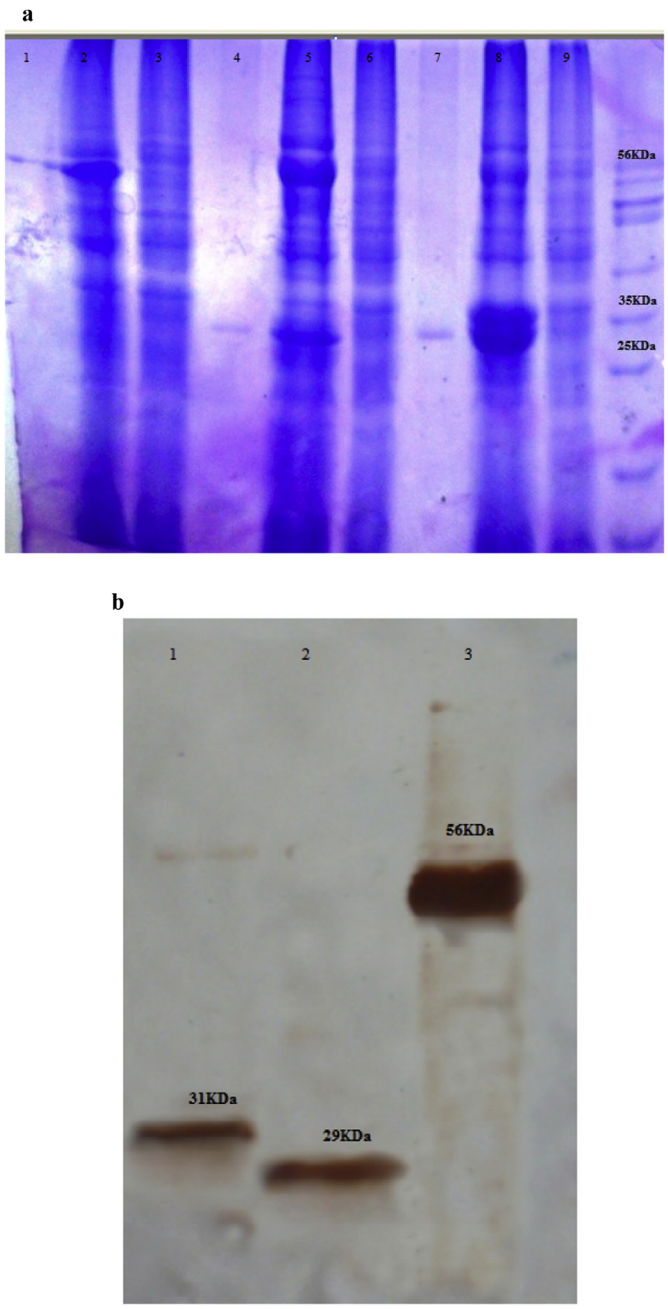

Fig. 1. a. SDS-PAGE analysis of purified recombinant K, B and C proteins. Column 1: 56 kDa protein K purified by Ni-NTA column; Column 2: Induced total protein k; Column 3,6,9: Uninduced total Protein as control K,B,C respectively; Column 4: 31 KDa protein B purified by Ni-NTA column; Column 5: Induced total protein B; Column 7: 29 kDa protein C purified by Ni-NTA column; Column 8: Induced total protein C. b. Western blotting of purified recombinant K, B and C proteins. Column 1: 31 kDa protein B; Column 2: 29 kDa protein C; Column 3: 56 kDa protein K.

showed 145% biofilm formation. The biofilm formation by high hydrophobic recombinant strains was higher than those of low hydrophobic strains. Fig. 6 shows biofilm formation by *A. baumannii* (76%), *E. coli* BL21 (DE3) (56%) and mFhaB1C1 (49%). The difference in the percentage of bacterial adhesion to p-xylene revealed hydrophobic characteristic of FhaB1C1. As shown in Fig. 6 FhaB1C1 exhibited a hydrophobic character with 62% affinity to p-xylene compared to mFhaB1C1 (8.7%), the *A. baumannii* (44%) and *E. coli* BL21(DE3) (16%).

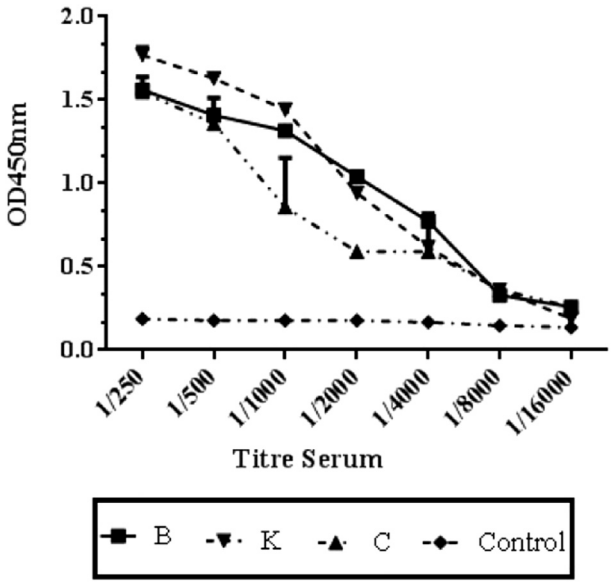

Fig. 2. Purified B, C and K proteins. Mean values ± S.E are from three independent replicates and values superscripted by Duncan's multiple range test ($P < 0.01$).

3.6. Hemolytic and hemagglutination assays

A variety of TPS exoproteins have been shown to lyse red blood cells when secreted from their native hosts. No hemolytic activity was observed on blood agar containing 5% sheep blood and 100 mM (IPTG + Arabinose) by FhaB1C1. *A. baumannii* and FhaB1C1 did not agglutinate human groups AB and O erythrocytes.

3.7. Animal challenge

In order to determine that the specific antibodies in immunized mice sera could reduce or prevent the *A. baumannii* infection,

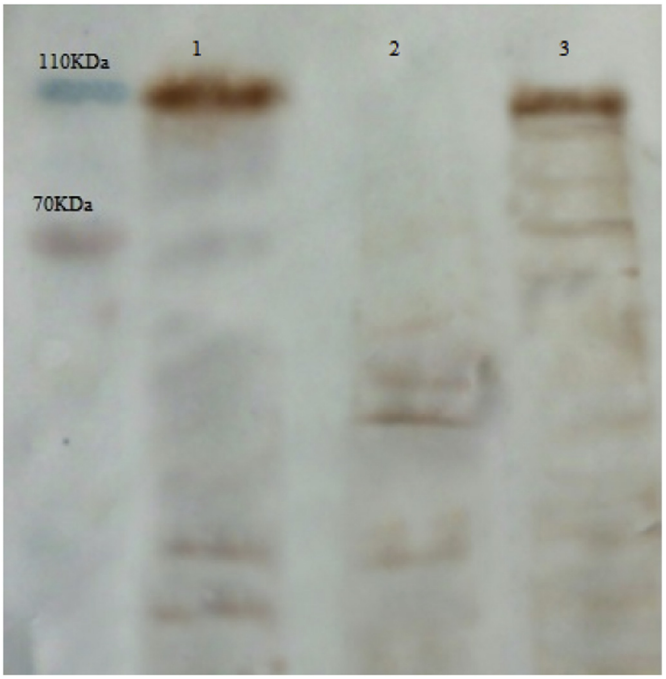

Fig. 3. Western blot analysis of FhaB1C1, mFhaB1C1 and *A. baumannii* whole cells. Column1:107 kDa TPS exoprotein FhaB1C1; Column 2: mFhaB1C1; Column 3: *A. baumannii*.

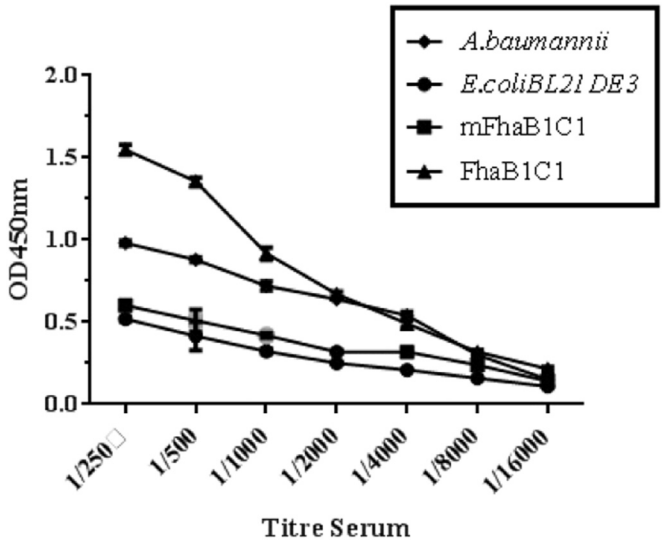

Fig. 4. Whole cell Eliza of FhaB1C1, mFhaB1C1, *E. coli* BL21DE3 and *A. baumannii* for FhaB1 location on outer membrane protein. Mean values ± S.E are from three independent replicates and values superscripted by Duncan's multiple range test ($P < 0.001$).

subcutaneously immunized and control mice were peritoneally injected with 10^{10} CFU of *A. baumannii* ATCC 190606^{T}. The unimmunized mice along with mice group immunized with the recombinant C protein died after 24 h. The mice immunized either with B or K recombinant proteins survived during a 7 day monitoring (Fig. 7a).

Subcutaneously immunized and non-immunized control mice were intraperitoneally injected with 10^{10} CFU of FhaB1C1 cells. 80% mortality was noted as compared to 100% survival in the immunized group until 5 days post inoculation in this animal model (Fig. 7b).

4. Discussion

Attachment to and biofilm formation on host cells is mediated by fimbria or membrane components. Subsequently they cause

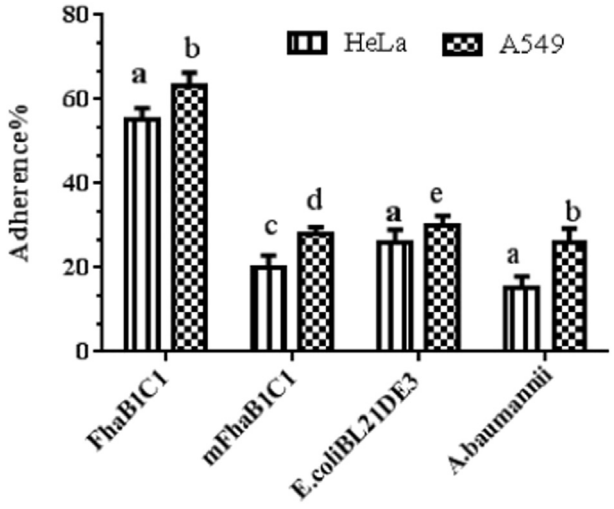

Fig. 5. In vitro adherence of the recombinant FhaB1C1, mFhaB1C1 and *E. coli* BL21DE3 and *A. baumannii* to A549 and HeLa monolayer cells. Mean values ± S.E are from three independent replicates. Values superscripted by different letters are significantly different by Duncan's multiple range test ($P < 0.001$).

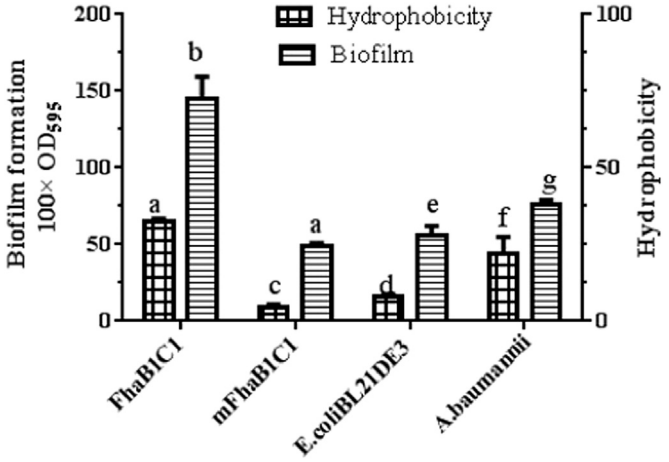

Fig. 6. Hydrophobicity and biofilm formation on an abiotic surface by *A. baumannii* and the recombinant FhaB1C1, mFhaB1C1, *E. coli* BL21DE3. Mean values ± S.E are from three independent replicates and values superscripted by different letters are significantly different by Duncan's multiple range test ($P < 0.001$).

various types of human infections including pneumonia, wound infections, urinary tract infections, bacteremia, and meningitis [26].

One of the factors playing a role in adhesion and biofilm formation is the TPS. The TPS is a pathway involving a single accessory

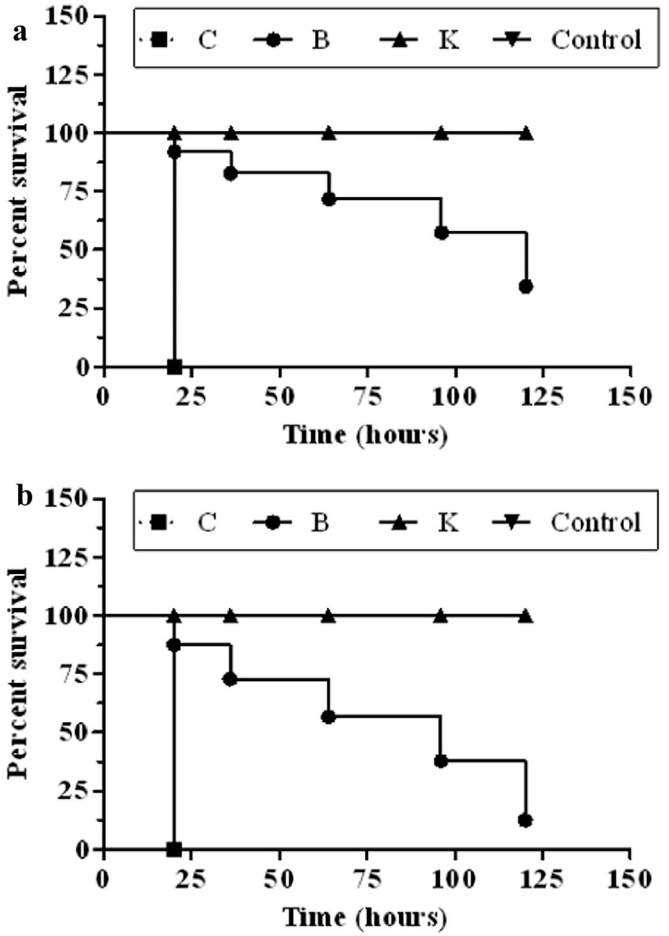

Fig. 7. a. Survival of mice immunized with *A. baumannii*. b. Survival of mice immunized with FhaB1C1. Chi square analysis with a 95% confidence interval showed that the immunized mice were significantly protected compared to the control.

protein i.e. TpsB, specifically devoted to the translocation of very large virulence proteins called TpsA across the outer membrane. TpsB proteins are part of a large family of channel-forming outer membrane porin-like proteins called Omp85 [15]. The TpsB domain of TPS in the OM of *B. pertussis*, FhaC, is a 64.4 kDa protein forming a porin-like structure that mediates the secretion and proper surface display of the FHA [27,28]. FhaC similarities were reported in other TpsB transporter proteins such as the MhaC of *Moraxella catarrhalis* (35%) [19] and OtpB of *E. coli* O157:H7 (26%) [29]. Our bioinformatic analyses also determined about 35% similarity of *A. baumannii* FhaC1 protein to other bacterial TpsB transporter homologues with an estimated molecular mass of 58 kDa (579 aa).

Together, these observations suggest structural and functional similarities between the *B. pertussis fha*C product and the *A. baumannii* ORFs. Thus, in the present study the ORFs were named *fha*C1 and *fha*C2 for *A. baumannii* FhaC-like proteins.

TPS domain subtypes harbor specific sequence motifs viz., NPFL (residues 143–146) and NPSGI (residues 183–187) found particularly in FhaB1. These motifs are similar to the conserved secretion motifs NPNL and NPNGI of *B. pertussis* FhaB, the prime candidates for the molecular interaction between TpsA and TpsB at the periplasmic side of the outer membrane during the secretion process. The analysis of the first 200 amino acids of FhaB revealed TPS location within this region. This conserved region of the TPS domain is a distinct feature for transporting the TpsA protein. The amino acid sequences corresponding to these large ORFs used as queries for BLAST searches (NCBI) revealed sequence similarities to other bacterial TpsA proteins reported earlier (MhaA of *M. catarrhalis* (28–41% similar) [19] and OtpA of *E. coli* O157:H7 (45% similar) [29]. FhaB1 protein was produced in our laboratory as a full-length protein. A signal sequence cleavage site detected between aa 26 and 27 indicates secretary nature of the gene product in *A. baumannii*. Regions of similarity including both secretion and functional domains were also found in FhaB adhesin of *B. pertussis* (36%) [30], the MhaB adhesin of *M. catarrhalis* (34%), the hemolysin HpmA of *Proteus mirabilis* (31%) [19], and the high-molecular-weight adhesin HMW1A of nontypeable *Haemophilus influenzae* (26%) [31].

TPS exoproteins have lysed red blood cells upon secretion from their native hosts [32]. Clantin et al. [33] redefined a region corresponding to residues 1 to 130 of FhaB of *B. pertussis* as the TPS domain of TpsA proteins. A potential hemagglutination activity domain reported in the Protein Families Database (PF05860) was found in the N-terminal portions of FhaB1C1. No hemolysis or hemagglutination was observed on blood agar containing 5% sheep blood by our recombinant cell harboring FhaB1C1. Absence of hemolysis or hemagglutination may be explained by the existence or influence of other factors from the native organism.

Domains responsible for hemagglutination are found in a number of proteins including FhaB adhesins (*B. pertussis*) [28] and HMW1/HMW2 (*H. influenzae*) [31,34], and the large secreted proteins LspA1 and LspA2 of *Haemophilus ducreyi* [32]. These proteins belong to the TpsA family, which are exoproteins secreted in a two-partner secretion manner [24,25].

Based on these sequence analyses revealing similarities between *A. baumannii* FhaB1 and FhaC1 and other related TPS systems, we hypothesized that FhaB1 and FhaC1 function as TPS system contributing to binding of *A. baumannii* to human epithelial cells. Our results on bacterial adherence indicate that the FhaB1 proteins are adhesins specifically involved in *A. baumannii* attachment to A549 cells.

The results provide evidence that TPS exoproteins can be translocated across the plasma and outer membranes in two distinct steps. Hence, we expressed *fha*B1 and *fha*C1 under the control of two distinct promoters in order to show that protein secretion does not require an interaction between the two partners prior to or during the assembly of the transporter. These findings are in support of Choi and Bernstein [35] working on *E. coli* O157:H7 OtpA/OtpB TPS system. Our findings are good evidence for FhaB1 and FhaC1 being as OM proteins.

Although many TPS systems are organized in one operon, our results clearly indicate that clustering is not necessary for TpsA secretion. Since the disruption of FhaC1 led to FhaB1 entrapment in the periplasm. The vital role of FhaC1 in the secretion of FhaB1 is obvious. These results demonstrate that FhaC1 is required for the secretion of FhaB1, however simultaneous transcription of FhaB1 and FhaC1 is not essential for FhaB1secretion. Based on the homology of the N-terminus of FhaB1 to the TPS domains of other exoproteins, we predicted that this segment would be sufficient to facilitate secretion.

The *A. baumannii* TPS described in this work, shows many important similarities with that of *B. pertussis* as well as other known TPS. We sought to gain insights into the structural role of the recombinant FHA-like protein in biofilm formation in vitro, using abiotic surface. There has been a growing realization that hydrophobic interactions play a role in many microbial phenomena including microbial adhesion to soft host tissues. Hydrophobic interactions are considered important in a variety of microbial adhesion phenomena in both the open environment, as well as the host [21,36]. Cell surface hydrophobicity influences the rate and extent of attachment of microbial cells [37]. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing nonpolar nature of one or both the microbial cell surface and the substratum surfaces involved. As bacterial hydrophobicity of *A. baumannii* and the recombinant FhaB1C1are associated with bacterial adhesion and biofilm formation, the hydrophobicity of FhaB1C1 was determined by the affinity of cells to xylene. FhaB1C1 showed the highest hydrophobicity compared to the wild type. The adhesion role of FHA showed its emergence as a multipurpose virulence factor capable of mediating *B. pertussis* invasion of host cells and as immunomodulatory molecule [16]. The FHA contribution to the initiation and maturation of *B. pertussis* biofilm formation represented a versatile role played by a single bacterial virulence factor in the emergence of *B. pertussis* [16]. *B. pertussis* FHA immunogenicity was reported to be immunoprotective by three overlapping recombinant fragments produced from the immunodominant region of FHA [38].

In order to obtain polypeptides for antibody production, immunologically important conserved regions were selected from *fha*B. Based on our bioinformatics analysis these epitopes are common in the conserved regions of *fha*B1 *and fha*B2. Hence no specific B1 epitope could be traced for antibody production. However, the produced antibodies were assayed only with *fha*B1 antigens in ELISA and Western blotting tests. The same was also true with *fha*C. The results of our experiments demonstrated that mouse immunized with FHA protein reduced mortality as compared to the control groups. Immunization with a combination of recombinant B and K proteins proved protective against *A. baumannii*. On this base a bivalent recombinant protein K containing a truncated form of FhaB1 and FhaC1 proteins, could protect the animal against *A. baumannii* ATCC19606^{T}. A significant difference in the antibody titer was observed among sera of the immunized mice and control groups. The sera reactivity with *A. baumannii* showed anti-K antibodies bound to FhaB1C1. The results from ELISA and Western blot experiments using whole cell shows presence of lower amount of surface exposed FhaB1 antigens. The reaction of serum raised against B with FhaB1C1 could be attributed to the sequence identity in the N-terminal region of this protein.

The overall observations of this study suggest that sequence features of TPS transporters do not necessarily specify the function of their cognate exoproteins. It is likely that common sequence

motifs in transporters simply reflect common biochemical or structural properties of exoproteins that have not yet been recognized. Therefore, Fha-like protein plays a role in *A. baumannii* virulence as determined in the mouse lethal model of infection, promoting biofilm formation and mediating the adhesion of *A. baumannii* cells to epithelial cell line. These findings may help promote the development of novel therapeutic strategies to limit *A. baumannii*-associated morbidity and mortality.

5. Conclusion

FhaB1 and FhaC1 constitute part of TPS systems described for *A. baumannii* ATCC19606^T. The chimeric protein produced in this study triggered significant protection against *A. baumannii*. Our data provide a foundation for the role and the function of this system in the pathogenesis of *A. baumannii*. The results are in support of the role of filamentous hemagglutinin adhesion B1 in *A. baumannii* adherence and biofilm formation. The findings may be applied in active and passive immunization strategies against *A. baumannii*.

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