Filamentous hemagglutinin adhesin FhaB limits *A.baumannii* biofilm formation

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1. ABSTRACT

Increased resistance and survival, as well as immune evasion, play a significant role in the pathogenicity of *Acinetobacter baumannii*. Here, we report on the adhesion of the bacterium to epithelial cells and formation of biofilm on abiotic surfaces. We identified autotransporter (AT) genes that encode homologues (Fha-like) of the two-partner secretion system (TPS) in *A.baumannii* (ATCC19606) which we designate as FhaB (exoprotein) and FhaC (transporter) and demonstrate that these novel genes, are under the control of distinct regulatable promoters within either the same (FhaBC) or two distinct (FhaB and FhaC) cells. The expression of this gene in outer membrane protein (OM) showed them to be deficient in the adherence to A546 cells. FhaB is involved in hydrophobicity of *A. baumannii* ATCC19606 while FhaBC is associated with biofilm formation. The vaccinogenic potential of FHA-like proteins offers use of these targets as novel therapeutic strategies to limit *A.baumannii* associated morbidity and mortality.

2. INTRODUCTION

Acinetobacter baumannii colonizes skin, respiratory and oropharynx tracts. In the recent years, treating such infections is severely limited because *A. baumannii* is resistant to nearly all current antibiotics (1). In order to understand the virulence mechanisms for possible discovery of more effective control measures, further investigations are required. Extensive research

on the virulence potential of this pathogen may involve a number of impressive virulence factors1-2)). The most common factors include environmental signals such as metal cations, nutrient availability, presence of pili, outer membrane proteins and macromolecular secretions. Proteins, such as phospholipase D and phospholipase C enhance the bacterial toxicity. OmpA, a member of the outer membrane proteins (OMPs), has been contributed to the disease potential of the pathogen and induces mitochondrial dysfunction leading to mitochondrial swelling and formation of apoptosome (3). Adhesion to surfaces is generally mediated by fimbriae and AT proteins. ATs are often associated with virulence functions such as adhesion, aggregation, invasion, biofilm formation and toxicity. AT proteins are unique in their primary sequence containing all the information needed to traverse the outer membrane (4). The AT, or type V, secretion system is a dedicated protein translocation mechanism which allows the organism to secrete proteins to and beyond the bacterial surface (5). There are two major players in TPS systems, the secreted proteins collectively called TpsA proteins and their outer membrane partners collectively called TpsB proteins. The TpsA are large proteins with masses ranging between 100 and 500 kDa (6). They are also of medical importance as essential factors in a wide variety of diseases causing great financial cost in the developed countries and great mortality elsewhere (7). The present study describes expression, identification, and characterization of an FHA-like adhesin in the OM of *E.coli* BL21DE3. The genes encoding TPS are arranged as *fha*B1 and *fha*C1 in one Open Reading Frame (ORF) and *fha*B and *fha*C in the other. We, focusing on *fha*B and *fha*C system, performed a comparative study of FhaB using both FhaBC and FhaB clones. An *in vitro* study was conducted on the role of protein in biofilm formation and adherence to human epithelial cells.

3. MATERIALS AND METHODS

3.1. Bacterial strains, plasmids, Cell lines, and growth conditions

pET22b and pET28a were from Novagen (USA). pBAD33 was kindly donated by Prof. Harris D. Bernstein, National Institutes of Health, Bethesda. The A549 and HeLa cells were purchased as a complete culture system from Iranian Biological Resource Centre and propagated as defined in the instructions from the manufacturer. Human bronchial A549 and HeLa 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. *E. coli* BL21 (DE3) was used as a host for the cloning and heterologous expression experiments. The recombinant *E. coli* were cultured in medium containing 20 μg ml^{-1} Chloramphenicol or 70 μg ml^{-1} Kanamycin or 100 μg ml^{-1} Ampicillin as and where indicated.

3.2. Bioinformatic analyses

A number of parallel bioinformatic approaches were used to identify the candidate AT genes in *A.baumannii* ATCC19606 based on the available sequences in Basic Local Alignment Search Tools (BLAST) in the National Center for Biotechnology Information (NCBI) nucleotide database. The percent sequence identity was determined by BLASTP using the default setting without a filter. Sequence alignments and dendrograms were generated using the ClustalW method in the program Megalign (DNASTAR, Inc.). AT genes were searched for highly conserved AT domains using the Pfam database including the AT beta domain (http://pfam.sanger.ac.uk//family/PF03797) and the hemagglutination activity domain http://pfam.sanger.ac.uk family/ PF05860(. SignalP4 (http://www.cbs.dtu.dk/services/SignalP/) was used to identify potential signal peptide encoding regions of the predicted AT coding sequences.

3.3. Amplification and cloning of fhaB and fhaC

Genomic DNA from *A. baumannii* ATCC19606 was purified using the Invitrogen Easy DNA kit. Plasmid DNA was purified with the Vivandis Kit. Unless otherwise stated all PCR experiments were performed using Maxime PCR Pre Mix Kit (i-pfu) (Interon). *fha*B gene was amplified from the genomic DNA of *A. baumannii* ATCC 19606 with primers *Nde*I-FhaB–F (CTTA CATATGATGA ATAA AAA TCTT TATC GA ATC A TTTT) and *Xho*I-FhaB-R(CTTACTCGAGTTACA TAT T TCTC CAA AA G A AT AAAC), then digested with *Nde*I and *Xho*I and cloned into pET22b to generate pET22-B. *fha*C open reading frame was amplified with the oligonucleotide primers *Nde*I-FhaC–F GTA ACATA TGATG TTAA CTAA GAAC TTTA TAA CTT CTTC)/ *Sal*/1-FhaC-R(GTAAGTCGACTTAATAAGAGAGAT TGATACTAAAAC). The PCR fragments specifying *fha*C was cloned into pBAD33, yielding the construct pBAD33-C.

3.4. Construction and purification of fusion protein

A bivalent recombinant chimeric protein "K" composed of conserved immunogenic regions of *fha*B and *fha*C was constructed and used for antibody production. Two functional regions namely "B" and "C" were focused. "B" was from the encoding epitopes of *fha*B comprising the residues 51 to 325 of the N-terminus of FhaB. Protein "C" was composed of 200 amino acids from the C-terminal of FhaC (residues 375 to 578). The *k* gene was synthesized with codon bias of *E. coli* for high-level expression. A bivalent protein was generated

using (EAAAK) linker within *B* and *C* genes. For more flexibility and efficient separation (8) four repeated sequences of EAAAK were introduced between two domains of B and C. The gene encoding target protein verified by Gen-Script (NJ, USA) and synthesized by Pars Biomatic (Iran) was delivered to us in the pUC57 cloning vector. The gene was sub cloned into pET28a and the construct is called pET-K. The similar strategy was used in the construction of the plasmids pET-B, pET-C. The recombinant proteins individually expressed in the *E. coli* BL21 (DE3). The cells were sonicated and suspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M urea) to separate inclusion bodies. The proteins were purified under denaturing conditions using the His-Bind resin system (Novagen). The protein was dialyzed at 4°C while gradually decreasing its concentration (6M>4M>2M>0M) over a period of 8 hours to remove urea.

Production and titration of polyclonal antibodies

Three mice groups received three doses of 20 µg from each of purified K, B and C proteins at 2 week intervals. The first dose of each vaccine was emulsified with complete Freund's adjuvant (Sigma) and the next two doses with incomplete Freund's adjuvant (Sigma). Blood samples were collected 15 days post-injection through infra-orbital plexus. An additional BALB/c mice group received PBS only served as control.

For antibody titration, a 96-well ELISA plates (Nunc) were coated with 20µg of K, B and C proteins in 100µl coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.8.). The plate was then incubated overnight at 4°C. To block the unoccupied sites, wells were washed once with PBS plus 0.0.5% Tween 20 (PBS-T) and then incubated with 100 µl of PBS-T plus 5% skimmed milk for 1 h at 37°C. After washing the plates 3 times with PBS-T (100µl per well), serial dilutions of each serum B, C and K from 1:250 to 10^{-4} 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 to 10^{-3} in blocking buffer) was added and the plates were incubated for 1 h. Plates were then washed 3 times with PBS-T and incubated with 100 µl per well of TMB for 15 min at room temperature. The reaction was stopped with 100µl of 2M H_2SO_4 and the absorbance was read at 450 nm.

3.5. Expression of FhaB and FhaC

In order to obtain evidence that FhaB and FhaC function as TPS, the two proteins were produced independently within the same cell. The pET22b-B harboring *fha*B (5800bp) and pBAD33-C harboring *fha*C (1740 bp) were transformed into BL21 (DE3) for the induction of FhaB under the control of an IPTG-inducible promoter and pBAD33-C encoding FhaC with a promoter under the control of arabinose. The cell harboring both pET22b-B and pBAD33-C plasmids were named as FhaBC and the one containing only pET22b-B was named FhaB. The cultures were induced with one or both inducers and incubated for 3h and then harvested by centrifugation. The cell pellets and culture supernatants were then probed by Western blotting and whole bacterial cell ELISA with anti-B antisera produced by polyclonal antibodies.

3.6. Localization of FhaB and FhaC on OM

Logarithmic growth (OD_{600}=0.5.) were used in the experiments. Plates coated with 100 µl of bacterial suspension per well were incubated overnight at 4°C and dried at 37°C. After blocking with 200ml of PBS containing 5% skimmed milk for 1 h, the plates were washed five times with PBS and incubated with100 µl of anti K serum (diluted to 10^{-3} in PBS) for 1 h. The plates were then washed and incubated with 100 µl of goat anti-mouse IgG peroxidase conjugate (Sigma) diluted to 10^{-3} in PBS, for 1 h. Plates were washed 5 times with PBS and then incubated with 100µl per well of 3,3',5,5'-tetramethylbenzidine (TMB) as substrate until a desired absorbance was reached. The reactions were stopped with 2 M H_2SO_4. The optical density at 450 nm was read on ELISA plate reader. The same procedure was applied for *E. coli* BL21 (DE3) and *A. baumannii*

3.7. Adherence assay

In vitro adherence of the recombinant FhaBC and *E. coli* BL21 (DE3) were compared with *A. baumannii* ATCC 19606 and FhaB using A549 and HeLa monolayer cells following methods of Balder and Plamondon (9-10).

Hemagglutination assay

Hemagglutinating activity of *A.baumannii,* FhaBC, FhaB and *E. coli* BL21(DE3) were determined by micro-hemagglutination test using 96-well round-bottom plates and fresh human O and AB groups, Rh positive erythrocytes (11).

3.8. Hemolytic assay

The hemolysis was performed on blood agar plates. FhaBC and FhaB were streaked on blood agar containing 5% sheep blood, 100mM IPTG and 0.2% arabinose with the appropriate antibiotics at 37°C (12).

3.9. Biofilm formation assay

Biofilm formation assays were performed with minor modifications of the method described by Brossard and Campagnari (13). Each assay was
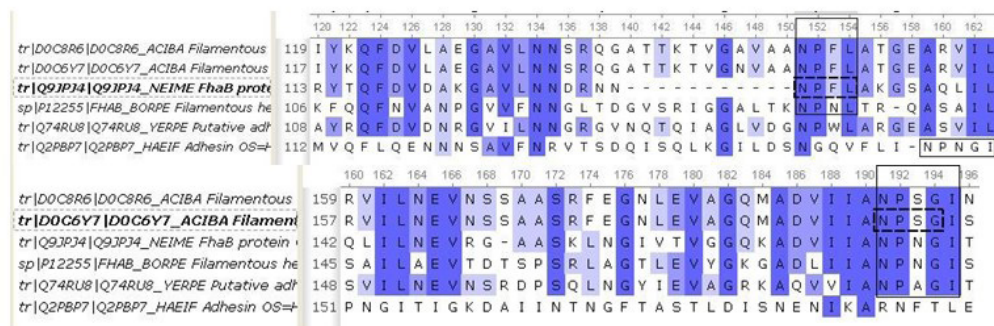
Figure 1. ClustalW alignment of conserved residues at the N termini of TPS exoproteins. Residues shadowed in blue match the consensus sequence. Brackets denote conserved NPFL and NPSGI motifs. tr|D0C8R6 and tr|D0C6Y7 Filamentous hemeagglutinin adhesion protein (*fha*B1, *fha*B2) in *Acinetobacter baumannii* ATCC 19606; tr|Q9JPJ4 |*fha*B in *Neisseria meningitides*; sp|P12255| FHAB_ BORPE in *Bordetella pertussis* (strain Tohama I / ATCC BAA-589); tr|Q74RU8| Q74RU8_YERPE Putative adhesin in *Yersinia pestis*; tr|Q2PBP7| Q2PBP7_HAEIF Adhesin in *Haemophilus influenza*.
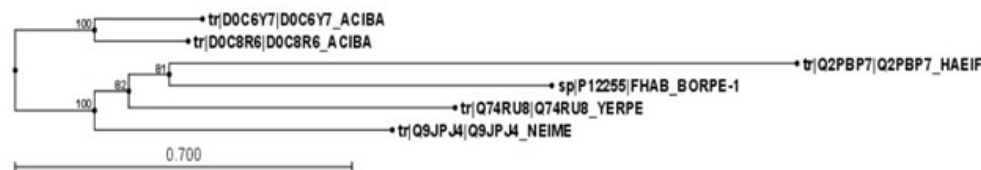

Figure 2. Phylogenetic tree showing the relationship between the selected TPS exoproteins. tr|D0C8R6 and tr|D0C6Y7 Filamentous hemeagglutinin adhesion protein (*fha*B1, *fha*B) in *Acinetobacter baumannii* ATCC 19606; tr|Q9JPJ4 |*fha*B in *Neisseria meningitides*, sp|P12255|FHAB_BORPE in *Bordetella pertussis* (strain Tohama I / ATCC BAA-589); tr|Q74RU8|Q74RU8_YERPE Putative adhesin in *Yersinia pestis*; tr|Q2PBP7| Q2PBP7_HAEIF Adhesin in *Haemophilus influenza.*

performed in triplicate. The OD_{595}<1 were considered as non-biofilm forming and OD_{595}>1 as biofilm-forming. For better resolution on plotting graph, the OD_{595} readings of the biofilm formation test were multiplied by 100.

3.10. Bacterial hydrophobicity

Bacterial cell-surface hydrophobicity was determined using a standard microbial adhesion to hydrocarbon (MATH) test (14). All the assays were conducted in thrice using fresh samples each time.

3.11. 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<0.0.5 was considered as statistically significant. The Graph pad prism version No 6 was used for plotting the graphs.

4. RESULTS

4.1. Bioinformatic analysis

FhaB was produced in our laboratory as a full-length protein. Analysis of the genomic sequence of *A.baumannii* ATCC 19606 using tblastn (NCBI) identified signal sequence cleavage site between aa76

and 77(VYA▼DI) indicates that the gene product in *A.baumannii* is secretary. Further analysis of the TPS domains of FhaB revealed an N-terminal extension linked to the signal peptide. The molecular weight of *A.baumannii* FhaB was estimated as 190 kDa (1898aa) resembling FhaB (expect value, 2e-31), the precursor of the adhesin FHA from *B. pertussis* BP536 (GenBank accession no.AAA22974). TPS domain subtypes harbor specific sequence motifs viz., NPFL (residues 143 to 146) and NPSGI (residues 183 to 187) found particularly in FhaB (Figure 1). Phylogenetic analysis shows that this highly conserved segment of FhaB is most closely related to the TPS domains of the FHA (Figure 2). The sequence analysis of downstream of *fha*B revealed a 1.7.kb ORF in the *A.baumannii* genome as in European Nucleotide Archive (ENA). ORF was 25% similar to the *fha*C gene of *Bordetella pertussis* Tomaha1 (NP_880575.1. GenBank). The *fha*C gene of *A.baumannii* ATCC19606 was amplified and sequence analysis predicted a 58-kDa protein of 579aa. Using SignalP 4.0., a putative signal sequence cleavage site was detected between residues 23 and 24 (AYA▼ID).

4.2. Expression, purification and immunogenicity of the chimeric protein

In order to obtain anti K, B and C antibodies, the chimeric protein was induced with 1mM Isopropyl-
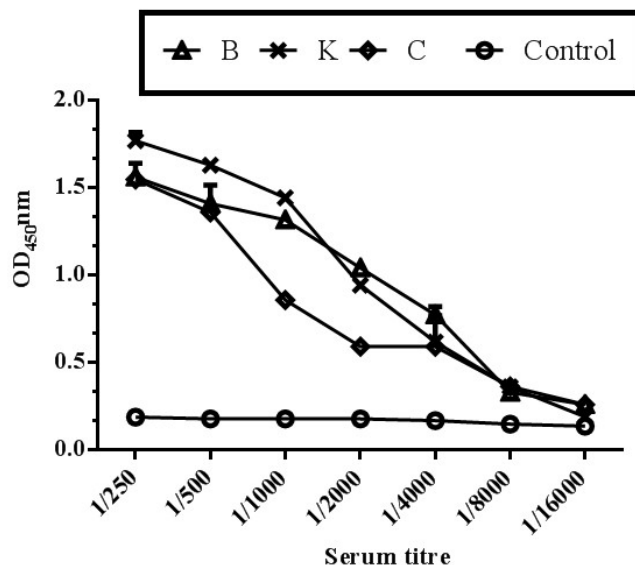
Figure 3. Antibodies raised in mice immunized with purified B, C and K proteins. Mean values ± S.E are from three independent replicates calculated by Duncan's multiple range test showing significant rise of antibodies compared to the control (P < 0.0.01).
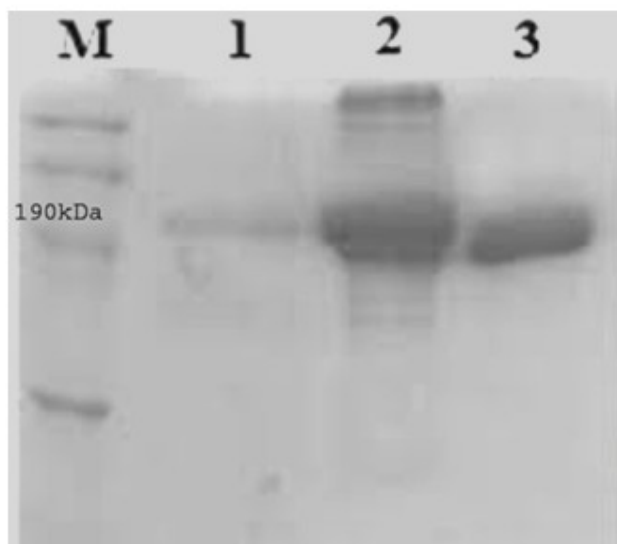

Figure 4. Western blot analysis of the recombinant protein from cells harboring FhaBC, FhaB and *A.baumannii*. Column1: A 190 kDa band of TPS exoprotein *A.baumannii*; Column 2: FhaBC cell; Column 3: FhaB cell.

d-galactopyranoside (IPTG) at OD 600 of 0.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed the presence of 56kDa, 31kDa and 29 kDa bands pertaining to K, B and C respectively. The recombinant proteins were then confirmed by Western blotting using anti His-tag antibody. Mice immunized with B, C and K proteins showed a significant rise of antibodies as compared to the control. The antibody titer increased significantly (P<0.0.1), whereas animals received adjuvant and

PBS, as the control had no specific antibodies in serum (Figure 3). *Expression and localization of FhaB and FhaC*

A prominent 190 kDa band was detected by anti-mouse antisera generated anti-K epitopes (Figure 4). Whole cell ELISA analyses exhibited the presence of FhaB and in the recombinant bacterial OM. FhaB and FhaBC had significantly (p<0.0.01) elevated levels of anti-B (Figure 5).
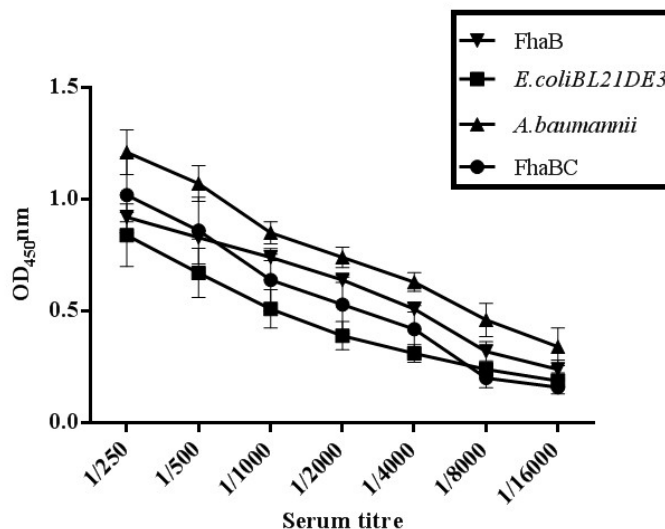
Figure 5. Whole cell Eliza analysis for FhaB location on outer membrane protein in FhaBC, FhaB, *E.coli* BL21 (DE3) and *A.baumannii.* Mean values ± S.E are from three independent replicates calculated by Duncan's multiple range test (P < 0.0.01).
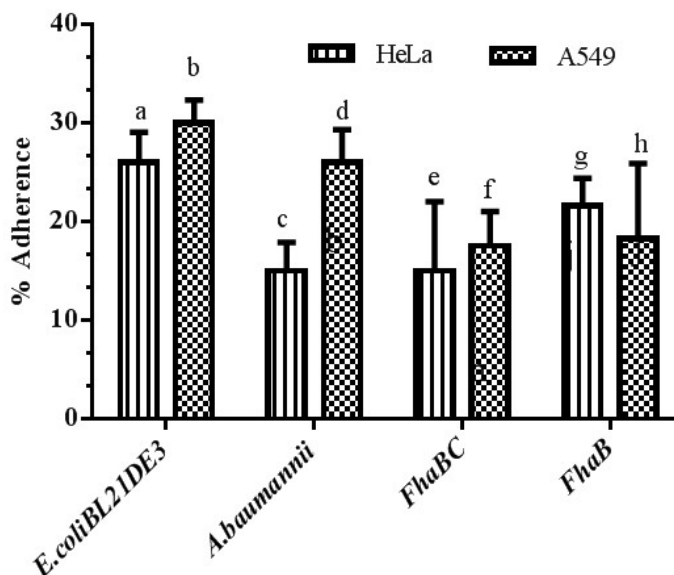

Figure 6. *In vitro* adherence of the recombinant FhaBC, FhaB, *E.coli* BL21 (DE3) and *A.baumannii* to A549 and HeLa monolayer cells. Mean values ± S.E are from three independent replicates and values superscripted by different letters are significantly different by Duncan's multiple range test (P < 0.0.01).

4.3. Adherence assay

To investigate as to whether FhaBC are involved in the initial colonization process, adherence of *A. baumannii* 19606, FhaBC, FhaB and *E.coli* BL21 (DE3) to human epithelial cells were compared *in vitro*. Figure 6 shows FhaBC significantly reduced binding to HeLa (15%) and A549 (17٪) epithelial cells compared to *A.baumannii* ATCC19606 (15% and 26%, respectively), FhaB (21% and 18%, respectively) and *E.coli* BL21DE3 (26% and 30%, respectively).

4.4. Hemolytic and hemagglutination assays

No hemolytic activity was observed on blood agar by FhaBC, *A. baumannii* and FhaB cells. Hemagglutination was also absent by the above strains.

4.5. Biofilm formation and Hydrophobicity assays

The ability of recombinant bacteria to form biofilm is a trait closely associated with bacterial persistence and virulence. We, therefore, investigated
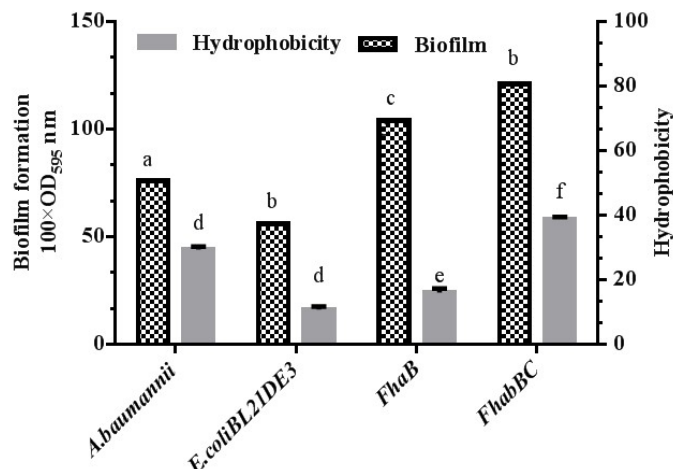
Figure 7. Hydrophobicity and biofilm formation on an abiotic surface by FhaBC, FhaB, *E.coli* BL21 (DE3) and *A.baumannii*. Mean values ± S.E are from three independent replicates and values superscripted by different letters are significantly different by Duncan's multiple range test (P < 0.0.01).

the role of FhaB in biofilm formation by FhaBC, FhaB, *A. baumannii* and *E.coli* BL21DE3. Results presented in Figure 7 show 121% biofilm formation by FhaBC as against FhaB (104%), *E.coli* BL21DE3 (56%) and *A.baumannii* (87%)(P<0.0.01). Correlation of hydrophobicity with biofilm formation on an abiotic surface is shown in Figure 7. The hydrophobicity of FhaBC (58%) was significantly (P<0.0.01) higher than those of FhaB (24%) (Fig.7).

5. DISCUSSION

Bacterial pathogens secreting virulence proteins serve many divergent functions in order to adhere, survive, multiply and disseminate within mammalian hosts(5). High multidrug resistance level observed in *A. baumannii* is attributed to active antibiotics and antiseptics transport mediated by efflux proteins (15).Virulence factors assigned for the translocation mechanism are known as the type V secretion system. In this system, proteins are secreted out of cells without the mediation of other proteins(16). Two-partner secretion system is a pathway involving a single accessory protein that each TpsB appears dedicated to transporting its cognate TpsA partner. TpsA homologues are known to play an important role in infection processes. TpsB proteins are part of Omp85, a large family of channel-forming outer membrane porin-like proteins(17). Bioinformatic analyses determined the similarity of *A.baumannii* FhaC protein to other bacterial TpsB transporter homologues such as MhaB of *Moraxella catarrhalis* (35%)(10) and OtpB of *E.coli* O157:H7 (34%) (12). There are structural and functional similarities between the *B. pertussis* FhaC and *A.baumannii* FhaC.

FhaB analysis revealed similarities to regions in TpsA protein in terms of both secretion and functional domains. FhaB was found similar to FhaB adhesin of *B. pertussis* (27%) (18), MhaA adhesin of *M.catarrhalis* (29%) (10), hemolysin HpmA of *Proteus mirabilis* (31%) (19) and high-molecular-weight HMW1A adhesin of untypeable *Haemophilus influenzae* (26%)(20).

Unlike long, polymeric, hair-like pili, autotransporter adhesins (ATADs) are nonfimbrial (16). Receptors of adhesive ATs have corresponded to one or more extracellular matrix proteins such as collagen, fibronectin, or vitronectin (21). Several adhesive ATs repeated Proline rich regions (PRR) which could represent binding sites. They have been suggested to restrict backbone flexibility and promote fast-acting weak binding (22). Nevertheless, adherence is an important initial step in host pathogen interactions and therefore strains with a relatively high adherence capacity are interesting subjects to study this interaction at the molecular and genetic level (23). In our study, the differences in quantitative adherence of FhaB and recombinant FhaBC strain are related to differences in the surface structures. We hypothesized that FhaB and FhaC function as TPS system contributing to the deficiency in adherence of *A. baumannii* ATCC19606 to human epithelial cells.

A 107 kDa AT protein known as Sat (secreted autotransporter toxin), a virulence determinant in *E. coli* CFT073, exhibited serine protease activity as well as cytopathic effects on VERO, HK-2 and HEp-2 cells. These phenotypes suggested that Sat may be a tool by which uropathogens promote infection (24).

It was shown that CsuA/BABCDE-mediated pilus plays a role in biofilm formation on abiotic surfaces. *A. baumannii* ATCC19606 produces a CsuA /BABCDE-independent thin and short pilus, which is not involved in adherence of *A. baumannii*ATCC19606

to bronchial epithelial cells (25). Hydrophobic interactions play a role in many microbial virulences including microbial adhesion (14). FHA with its adhesion role as an immunomodulatory molecule is the main virulence factor of *B. pertussis* (26). The cell surface hydrophobicity has been shown to be important in biofilm formation as hydrophobic interactions tend to increase with increasing non-polarity of one or both the microbial cell surface and the substratum surfaces (27). Bacterial hydrophobicity of FhaBC and FhaB assessed by measuring their affinity to xylene exhibited FhaBC with the highest hydrophobicity as compared to the wild type and FhaB.

Clantin *et al*. (2004) re-defined a hemagglutination domain of *B.pertussis* as the TPS domain of TpsA proteins. These proteins belong to TpsA family in a two-partner secretion manner (28-29). A potential hemagglutination activity domain was found in the N-terminal portions of the amino acid sequences of FhaBC in the Protein Families Database (PF05860). Absence of hemolysis or hemagglutination may be explained by the existence or influence of other factors of the native organism.

Although many TPS systems are organized in one operon, our results clearly indicate that clustering is not necessary for TpsA secretion. We separated *fha*B and *fha*C under the control of distinct promoters. Secretion of FhaB is through the FhaC protein across the OM. The FhaB gain its final mature structural conformation at the cell surface. Our findings provide good evidence for FhaB and FhaC location in OM. Since absence of FhaC leads to FhaB entrapment in the periplasm, it can be stated that FhaC is required for the secretion of FhaB, however, simultaneous transcription of FhaB and FhaC were not essential for FhaB secretion. Western blot analysis and whole cell ELISA showed the presence of a lower amount of exposed FhaB antigens in FhaB cell. The sera reactivity suggested the binding of anti-B antibodies to FhaBC and FhaB. The reaction of serum raised against anti-B with FhaBC could be attributed to the sequence identity in the N-terminal region of this protein. Based on the homology of the N terminus of FhaB to the TPS domains of other bacteria as well exoproteins, we predict that this segment is sufficient to facilitate the secretion.

A.baumannii TPS described in this work resembled many important similarities with that of *B. pertussis* as a well-known TPS(30). TpsA can be released in part by interaction with the serine protease SphB1(29). These findings are in support of Choi and Bernstein(31) working on *E. coli* O157:H7 OtpA/OtpB TPS system. AT proteins constitute an essential component of some human vaccine (4-32).

Our studies suggest that FhaB and FhaC could constitute *A.baumannii* TPS system. The findings are in support of FhaB homology to FhaB of *B. pertussis*, a critical component of the new version of the acellular pertussis vaccine against whooping cough. Our data provide a foundation for further studies designed to determine the role and the function of this TPS in the pathogenesis of this important human pathogen. The present results favor the possibility of exploitation of homology phenomenon in the application of FhaB as a protective immunogen against *A.baumannii*.

6. ACKNOWLEDGEMENTS

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