ADHESION OF *ACINETOBACTER BAUMANNII* TO EXTRACELLULAR PROTEINS DETECTED BY A LIVE CELL-PROTEIN BINDING ASSAY

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Introduction: *Acinetobacter baumannii* is involved in various infectious diseases ranging from nosocomial community-acquired infections to those acquired following war or natural disasters. The treatment has become exceedingly difficult partly because the bacterium can form biofilms. Therefore, it is imperative to elucidate mechanisms of the biofilm formation that may be exploited to develop therapeutic strategies.

Objective: To develop an assay by which the role of the bacterial extracellular proteins can be studied in mediating cell adhesion and biofilm formation.

Methods: Biofilm mutants of *A. baumannii* were generated. Proteins from the cell-free spent cultures and outer membrane fractions of the mutants and the wild type strain were characterized by SDS-PAGE based proteomic analysis. The PAGE-based membrane binding assays were developed to examine bacterial adhesion to the released proteins immobilized on the blotting membranes.

Results: The mutants exhibited deficiencies in formation of biofilms and in production of the biofilm-related proteins, such as OmpA. A novel PAGE-based membrane binding assay was established, and the results show attachment of the wild type cells to the released proteins in contrast to that of the mutant cells deficient in the outer membrane proteins. The results imply that these mutants have lost the cell surface-associated proteins that mediate cell adhesion to the released proteins.

Conclusion: This novel assay can be used to study the live bacterial adhesion to extracellular proteins. The results suggest that the outer membrane proteins may mediate cell attachment through binding to the released proteins for biofilm formation. (*Ethn Dis.*2010;20[Suppl 1]:S1-7–S1-11)

Key Words: *Acinetobacter baumannii*, Infections, Bacterial Biofilms, Proteins, Adhesion

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INTRODUCTION

Acinetobacter baumannii is an emerging nosocomial pathogen. As a Gram-negative, obligate aerobic and nonmotile bacterium, it can be isolated from soil, water, sewage, and healthcare settings. It is associated frequently with a wide spectrum of infectious diseases ranging from nosocomial, community-acquired infections to those acquired following war or natural disasters. It causes mild-to-severe illness of various types: post-surgical urinary tract and respiratory tract infections, nosocomial pneumonia and bacteremia; some of which can be fatal with mortality rates as high as 75%.[1] These nosocomial infections often result from many events, such as surgery, application of artificial devices and exposure to broad-spectrum antibiotics.

Options for treating such infections are severely limited because the bacterium is capable of developing extensive antimicrobial resistance, and *A. baumannii-calcoaceticus* complex is resistant to nearly all current antibiotics. Above all, bacterial cells can attach to medical devices and host cells[2] and form biofilms.[3] Biofilms are multi-cellular communities of bacterial cells[4] that contribute to drug resistance[5] and chronic infections.[6] Antimicrobial agents can even induce biofilms.[7–9] Alarmingly, for such an emerging pathogen as *A. baumannii*, little is known about the fundamental mechanisms of the biofilm formation. It is imperative to elucidate the molecular mechanisms that can be exploited to develop therapeutic strategies. The purpose of our study was to develop an assay of live-cell adhesion to extracellular proteins by which the role of extracellular proteins of *A. baumannii* could be investigated in mediating bacterial adhesion and biofilm formation.

METHODS

Strains and biofilm assays

Polystyrene tubes containing LB broth were inoculated with overnight culture of *A. baumannii* strain ATCC 19606. The cells were grown under shaking and static conditions. Biofilms were stained with 0.1% crystal violet for 10 minutes and washed with water.

Mutagenesis

A standard approach was used to screen for the resistant mutants.[10] Specifically, two types of colony morphologies were observed on the LB agar plates (Fig. 2C). Colonies (200) of each type were inoculated into LB broth containing 5 mM hydroxyurea. The cultures that grew in 5 mM hydroxyurea and failed to form biofilm were subcultured 10 times with 5 mM hydroxyurea. The cultures were screened by plating on the 5 mM-hydroxyurea plates. The resistant mutants were examined for biofilm formation.

Cell binding assay

The SDS-PAGE and membrane blotting were performed according to Sambrook et al.[11] Membranes were blocked for 1 hr at 25°C with 3% (w/v) BSA, followed by three washes with PBS.

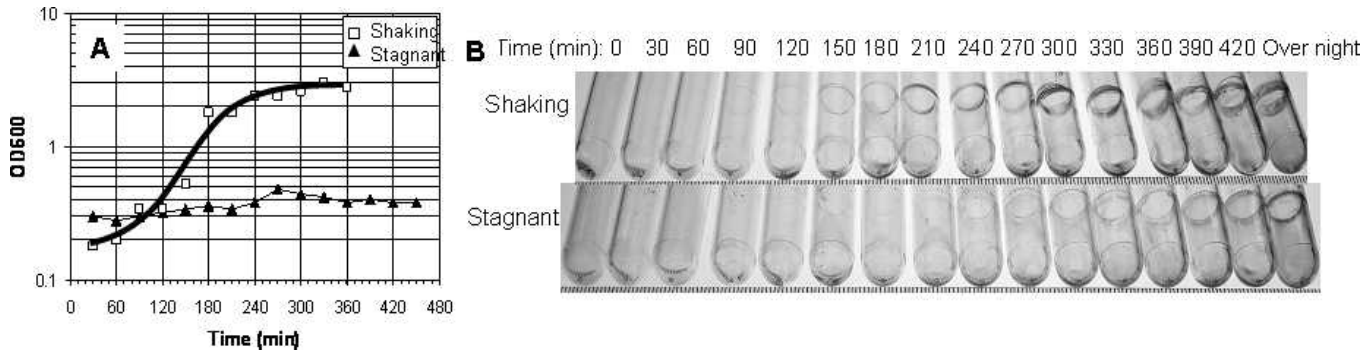

Fig 1. Planktonic growth and biofilms of *A. baumannii*. (A) Planktonic growth curves. (B) Biofilm formation in the shaking or stagnant Luria-Bertani (LB) cultures at 37°C

The membranes were incubated with *A. baumannii* (10^8 cells) in LB broth for 1 hr at 25°C. The membranes were then washed with PBS gently and the cells were fixed with 4% paraformaldehyde for 10 min at 25°C. The membrane was washed and stained with 0.1% amido black for 60 sec and destained with 45% methanol and 10% acetic acid.

RESULTS

Rationale and Experimental Design

A. baumannii forms biofilms[3,12] and biofilm formation generally entails cell attachment and matrix construction. The matrix contains polysaccharides, proteins and nucleic acids;[1] this work focused on the role of extracellular proteins in biofilm formation. While the mechanism for *A. baumannii* biofilm formation remains to be studied, we proposed that extracellular proteins of *A. baumannii* mediate the bacterial attachment. To test this hypothesis, we characterized *A. baumannii* biofilm formation. Then we performed mutagenesis of the wild type strain and screened the mutants for the phenotype of deficiency in biofilm formation. Further, we characterized the protein profiles of the mutants. Lastly, we conducted the functional study to elucidate the role of the extracellular proteins in mediating the bacterial attachment.

A. baumannii Biofilm Formation under Shaking and Static Conditions

To characterize *A. baumannii* biofilm formation, we first examined the growth behaviors under shaking and static conditions. As illustrated in Fig. 1A, the exponential phase of the planktonic cells in the shaking culture lasted approximately 4 hours with a generation time of 20 minutes, but was not observed in the static culture. Second, following the time course, we observed that biofilms formed as cellular rings at the air-medium interface in both shaking and stagnant cultures (Fig. 1B). The rings became visible earlier under shaking at 90 and 120 minutes than under the static growth condition at 240 and 270 minutes. We also conducted confocal laser scanning microscopy (CLSM) of the 24-h static biofilms forming on the glass surface as we previously described,[3,12] confirming the biofilms with confluent cell layer morphology (data not shown). These observations are consistent with the previous finding that *A. baumannii* forms biofilms in static cultures[3,12] but our results indicate that the biofilms also form in the shaking cultures.

Screening Mutants for Deficiency in Biofilm Formation

To generate mutants deficient in biofilm formation, we performed mutagenesis with DNA replication inhibitor hydroxyurea. Particularly, the minimal inhibitory and minimal bactericidal concentrations of hydroxyurea were determined, that is, 2 mM and 5 mM, respectively. The wild type strain was grown in the static cultures containing a wide range of hydroxyurea from 1 mM to 100 mM. The planktonic cells were susceptible to hydroxyurea (Fig. 2A), and biofilm formation was reduced as a function of the drug concentrations after a 24-hour incubation (Fig. 2B). When the cells (approximately 10^4) treated with 5 mM hydroxyurea were subcultured, the two types of colony morphologies with large and small size appeared (Fig. 2C). Approximately 200 colonies of each type were screened for resistance to 5 mM hydroxyurea, and all the colonies of both types were resistant. However, only 4 out of the 200 larger colonies (2% of the large colonies) did not form biofilms under shaking and non-shaking conditions (Fig. 2D). The resistant mutants are called HU^r biofilm mutants. The strain identity of all the mutants was confirmed with PCR-based DNA sequencing of the 16S ribosomal DNA[14] and *kdtA* genes.[13–15]

HU^r biofilm Mutants Deficient in OmpA and Other Proteins

To characterize the mutant phenotypes related to the role of proteins in

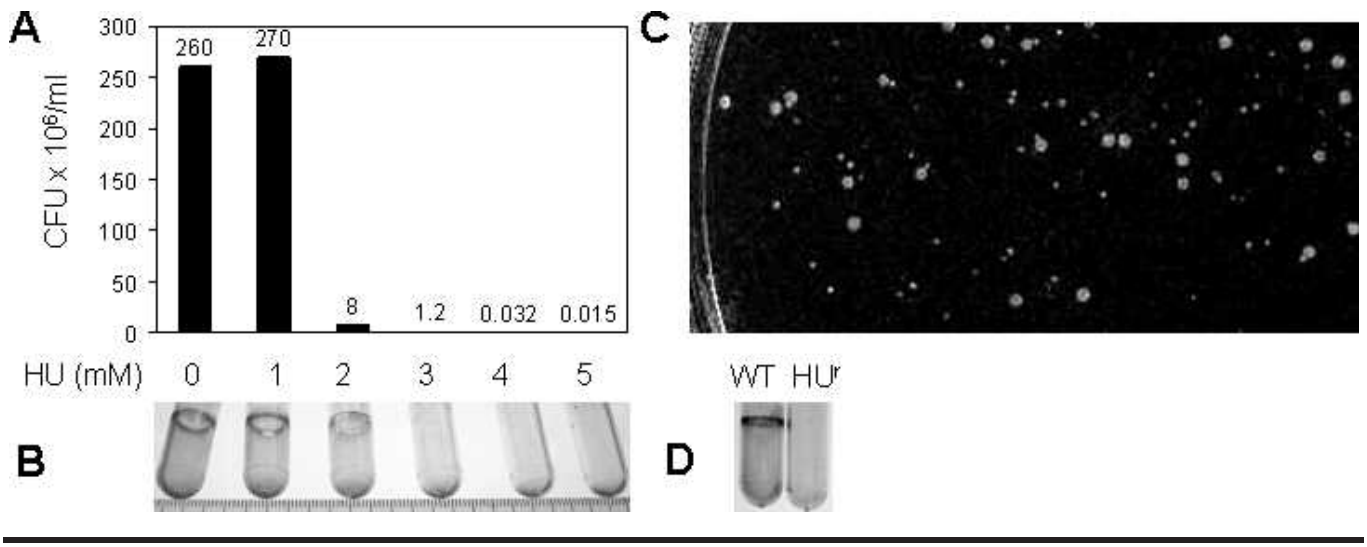

Fig 2. Generation of biofilm mutants in *A. baumannii*. (A) viability and (B) biofilms in LB broth containing hydroxyurea. (C) two morphology types of colonies growing on the LB agar plates. (D) biofilm formation of the wild type and the HU^r mutant

biofilm formation, we examined the proteins extracted from whole cell lysate, membrane fractions, and cell-free spent cultures of the wild type and the HU^r biofilm mutants. As shown in Fig. 3A, the whole cell protein profile of the HU^r biofilm mutant (Lane 3) appeared similar, to a certain extent, to that of the wild type (Lane 2). However, noticeable differences were observed in that a 49-kDa band was intense in the mutant (*in Lane 3), but most others were weaker. Such deficiencies were apparent in the membrane protein profile (Fig. 3 B, Lane 5 vs 4). For proteins from the mutant cell-free cultures (Fig. 3C), two bands (marked with arrows) appeared lower in intensity (Lane 8 vs. 7).

To identify these proteins deficient in the mutants, we selectively cut out the corresponding bands, as indicated by arrows from the wild type gels (Fig. 3B Lane 4) and performed capillary liquid chromatography-tandem mass spectrometry (LC/MS/MS) with protein database searching. The data showed that the major membrane proteins deficient in the HU^r biofilm mutant are a 37-kDa OmpA and a 17-kDa CsuA/B (Lane 5 vs 4). Other outer membrane proteins from the two bands marked in the middle of lane 4 have no

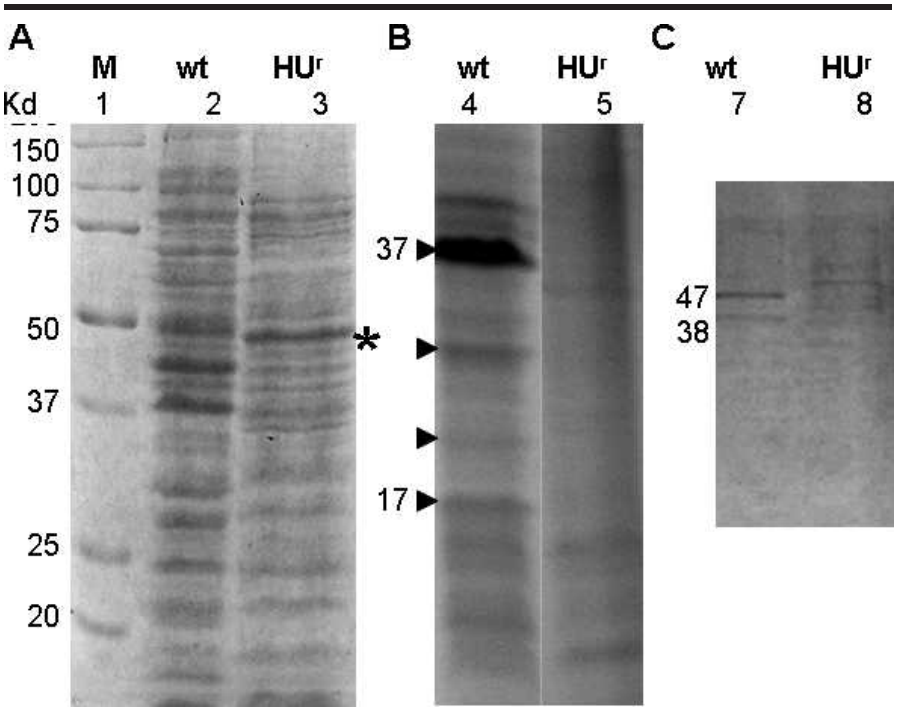

Fig 3. Protein profiles of *A. baumannii* proteins. (A) proteins from whole cell lysate separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). (B) proteins from membrane fractions resolved by 12% SDS-PAGE. The major proteins deficient in the HU^r biofilm mutant are OmpA (37 kilo-Dalton, kDa) and CsuA/B (17 kDa). (C) proteins from the cell-free cultures resolved by 12% SDS PAGE. Lane 1: Markers. Arrows: protein bands that were cut off from the gel for protein sequencing. Star: a 49-kDa strong band in the mutant

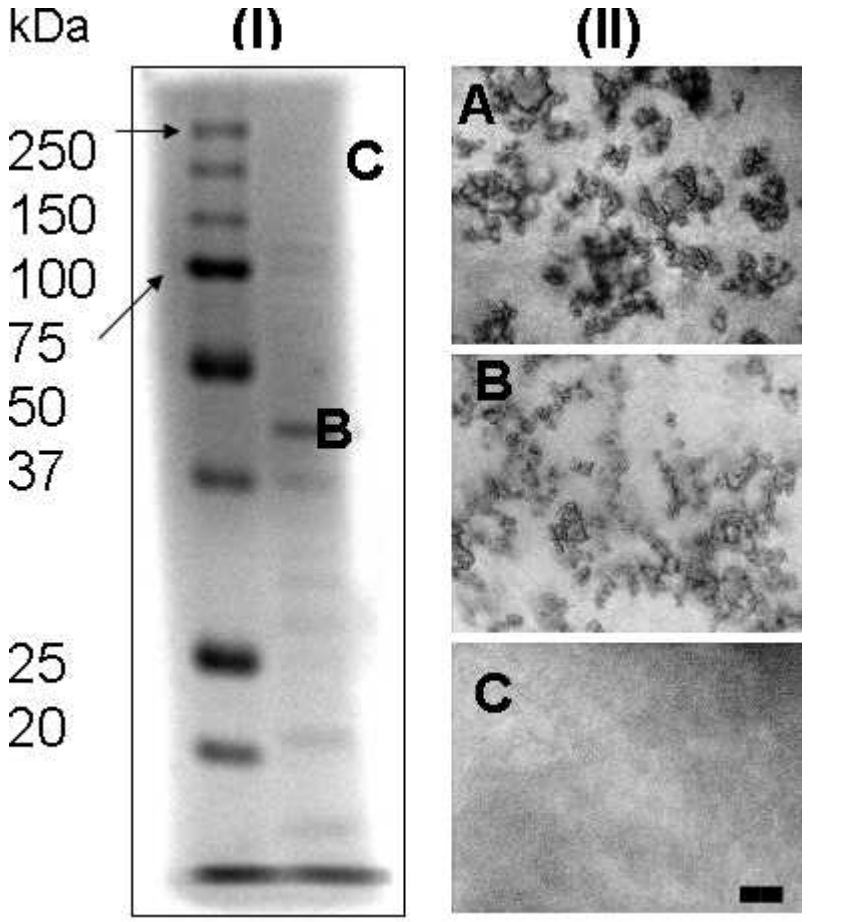

Fig 4. Live cell binding assay. Proteins extracted from the cell-free spent cultures were resolved by SDS–PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes, and the membranes were incubated with the wild type *A. baumannii* cells and washed afterward. As depicted in (I), B is the protein track, and C is the blank region. (II) Exponentially growing cells attached to the membrane were examined under a light differential interference contrast (DIC) microscope. A, the positive cell control with the live cells spotted on the membrane; B and C, the marked area as shown in (I). Bar, 10 μm

defined functions. Significantly, most of those proteins are involved in biofilm formation. Csu and Omp contribute to the *A. baumannii* biofilm formation.[12,16,17] The proteins deficient from cell-free cultures are under investigation.

A Novel Assay of Live Cell Adhesion to Immobilized Proteins

The HU^r biofilm mutants are defective in formation of biofilms and deficient in production of certain membrane proteins and released proteins. These observations led to an overarching hypothesis that the bacterial adhesion is mediated through binding of the membrane proteins to the released proteins immobilized onto the surface. Testing this hypothesis requires an assay of live cell adhesion to immobilized proteins. To this end, we developed the PAGE-based membrane binding assay. Briefly, the released proteins from the wild type spent culture were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with the wild type or the mutant cells, followed by washing off the unbound cells. The cells that had bound to proteins on the membranes were stained and examined under a light microscope.

As indicated by the assay, the wild type cells differentially adhered to the released bacterial proteins blotted on the PVDF membrane. We examined the differential adhesion by scanning under light microscope the cells on or outside the protein track. We observed that the wild type cells were mostly found within the bacterial protein track (Fig. 4IB and IIB) but marginal outside the track (Fig. 4IC and IIC). In sharp contrast, almost no cells were observed on the blank track or the track of the secreted proteins from budding yeast *Saccharomyces cerevisae*. Since cell clusters often formed noticeably on the bacterial protein track (Fig. 4IIB), we were unable to obtain the accurate cell number. Nevertheless, the results of cell clustering differentially on the bacterial protein track indicate that the wild type bacterial cells specifically adhere to the released bacterial proteins on the PVDF membrane and suggest that these proteins mediate cell attachment. Due to the limited resolution of SDS-PAGE, the protein bands that had captured the cells could not be determined; this problem is being investigated with the 2D gel-based adhesion assay combined proteomic analysis.

The HU^r biofilm mutants were examined the same way. They adhered scarcely to either the protein or the blank tracks (data not shown). The results indicate that the mutants defect in adhesion to the released proteins of the wild type cells. The data are consistent with the earlier observation that the mutants were deficient in biofilm formation. Since these mutants also are deficient in OmpA, the major protein in the outer membrane fraction (Fig. 3B lane 4), it can be hypothesized that OmpA contributes to mediating the adhesion through binding to the released proteins while the other proteins can not be excluded. This hypothesis is under further investigation by

using complementation of the mutants with the gene encoding OmpA and the 2D gel-based adhesion assay with antibodies against OmpA.

DISCUSSION

We isolated mutants of *A. baumannii* that have phenotypes of resistance to hydroxyurea and deficiencies in biofilm formation and in production of the biofilm-related proteins. We developed the PAGE-based membrane binding assay, showing attachment of the wild type cells to the released proteins in contrast to that of the mutant cells deficient in the outer membrane proteins. The results imply that these mutants may lose the cell-surface-associated proteins that mediate cell adhesion to the released proteins. These results advance the hypothesis as to whether the bacterial adhesion is mediated through binding of the outer membrane proteins to the released proteins.

Furthermore, testing this hypothesis should provide insights into understanding mechanisms of biofilm formation, at which immunization and therapeutics can be developed to disrupt *Acinetobacter* adhesion and biofilm formation as proposed previously.[18] For example, Omps are involved in *A. baumannii* biofilm formation[16,17]; among which, OmpA is one of the major proteins in the outer membrane. While OmpA is required for structural integrity of the outer membrane and for normal cell shape, it is also involved in invasion of *E. coli.*[19] Hence, OmpA seems to be good vaccine targets; in fact, *A. baumannii* OmpA activates dendritic cells and induces an alternative T helper 1 (Th1)-promoting interleukin-12 (IL-12), leading to stimulation of CD4 and production of interferon-γ (IFNg).[20] Although vaccines against *Acinetobacter* are under investigation, the hypothesis suggests that disruption of the OmpA and the released proteins by antibodies may block the bacterial adhesion and biofilm formation.

This study should lead to future research and development of effective immunization against *Acinetobacter* infections. Such a measure will contribute to reducing health disparities among the patients in the United States since recent studies showed that the majority of the battle casualties from ongoing campaigns in Iraq and Afghanistan had wounds that had been infected with *A. baumannii*.

ACKNOWLEDGMENTS

This work is supported by San Antonio Area Foundation.

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