Full Paper

Biotic and abiotic substrates for enhancing *Acinetobacter baumannii* biofilm formation: New approach using extracellular matrix and slanted coverslip technique

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Acinetobacter baumannii has been well recognized as a problematic human pathogen and several reports has shown the incidence of multidrug and pandrug-resistant *A. baumannii* strains in infirmity infections. *A. baumannii* grows only on an air-liquid interface and does not form a contiguous biofilm. Extracellular matrices (ECM) and slanted glass coverslips are (SGC) used as biofilm substrates and biofilms have been investigated by SEM, confocal and crystal violet staining. ECM has shown enhanced biofilm formation under dynamic conditions rather than static conditions. SGC biofilm yield assay has shown higher levels of continuous layers and packed thicker biofilm formation with glass coverslip inserts, up to 1.7 to 3 times higher biofilm formation, than when compared with no glass coverslip inserts. A media immersed ECM study revealed that biofilm grown on extracellular matrixes formed thread-like pili structures, and that these structures had contact with the ECM and also showed excellent cell-to-cell interaction. In summary, *A. baumannii* showed higher biofilm formation capacities with ECM, while the prominent results were directly related with the biofilm formation capacity of *A. baumannii*. For the initial step of biofilm formation, adherence is an important factor and, consequently, strains with a comparatively high capability to adhere to extracellular matrices and slanted glass coverslips provide a new method of enhanced biofilm growth for *in vitro* assays. ECM can be used as a substrate for immersed biofilm formation studies and the SGC

method for air-liquid interface exposed biofilm formation studies, and these substrates can provide better biofilm growth and easy handling for *in vitro* adherence and biofilm assays.

Key Words: *Acinetobacter baumannii*; biofilm growth; biofilm substrates; extracellular matrix

Introduction

Acinetobacter baumannii is well-recognized as a significant and problematic human pathogen, and several reports showing the incidence of multidrug and pandrug-resistant *A. baumannii* strains in infirmity infections have been reported (Dijkshoorn et al., 2007; Jeong et al., 2006). The prevention and control of this pathogen requires an effective and non-resistance-promoting antimicrobial agent. However, antimicrobial agents developed for use with *A. baumannii* are hindered by its biofilm growth pattern *in vitro*. This bacterium, a strictly aerobic bacterium, does not form adjoining cell layers of biofilm even after prolonged incubation (Tomaras et al., 2003), and, in addition, grows only at the air-liquid interface. Bacterial strains of *Acinetobacter* produce fragile adhesion and cohesion behavior on different surfaces; on some surfaces the bacteria produce very weak cohesion and no adherence patterns, with the exception of a glass surface (Costa et al., 2006; Egwari and Taiwo, 2004). This growth pattern (air-liquid interface) may cause stress to the bacteria, and in general, starvation for essential nutrients, e.g. carbon, phosphate and nitrogen (Harder and Dijkhuizen, 1983;

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Spector, 1998). According to Mihaljevic et al. (2007), nutrient availability (starvation) is the influential stress factor, which significantly affects bacterial cultivability and viability, and also affects adherence and incursion properties. Desiccation is another important factor, causing water loss through evaporation and affecting the survival and persistence of bacteria (Michael et al., 2012). Considering the above growth properties of *A. baumannii*, the purpose of this work was to examine different extracellular matrices and slanted glass coverslips as biofilm substrates to provide enhanced growth conditions to avoid stress in exposed cultures.

Materials and Methods

Bacterial strains and growth conditions. *Acinetobacter baumannii* strains (ATTC BAA-1605, 17961, 19606, 19003) were procured from ATCC (ATCC, Manassas, VA). BAA-1605 is a multidrug resistant strain isolated from the sputum of military personnel returning from Afghanistan in 2006 (Stuart et al., 2007). Strains 17961, 19606 and 19003 were isolated from blood, urine, and the cerebrospinal fluid of patients, respectively. Nutrient broth and agar (BD, Franklin Lakes, NJ) were used for bacterial culture. Cultures with OD 0.3–0.4 at 490 nm (10^7 CFU/ml) were used for all studies.

Quantification of biofilm yield using the slanted coverslip method. For the slanted glass coverslip (SGC) method, the coverslip (20 mm × 11 mm) extended above the level of the medium to about 1/3 of its length and allowed the formation of a biofilm to begin at the level of the upper surface of the medium. Thereafter, a contiguous biofilm grew both below (into the medium) and above this level, covering the entire coverslip. To evaluate the biofilm yield, overnight bacterial cultures containing 10^7 CFU/ml of *Acinetobacter baumannii* strains (ATTC BAA-1605, 17961, 19606, and 19003) were added to 2 ml of media in 24-well microtiter plates with, or without, inserted slanted glass coverslips. Every 12 h, the medium containing suspended bacterial cells was removed and an equal volume of fresh medium was added. After incubation, the glass coverslips, and the well plate with attached bacteria, were fixed with 2.5 ml of methanol per well for 15 min. The well plate with, and without, glass coverslips were then emptied and air dried. Each well was then stained for 2.5 min with 5 ml of 0.1% (w/v) crystal violet. Excess stain was rinsed off by placing the plate under running tap water. The well plate with, and without, glass coverslips was air-dried and the dye bound to adherent cells removed with 2.5 ml of 33% (v/v) glacial acetic acid per well. The optical density of the resulting solutions was measured at 595 nm. Three independent experiments were carried out for each experimental condition. In each case, the mean optical density of the negative control was subtracted from the readings of all other samples.

Live and dead biofilm assay for the slanted glass coverslips method. For the biofilm live and dead assay, biofilms were formed as described in the above biofilm yield assay. The glass coverslips with grown biofilm were stained using an L 7012 LIVE/DEAD BacLight Bacterial

Viability Kit from Molecular Probes Inc. (Eugene, OR) as described by Neu and Lawrence (1997). The samples were immediately examined via confocal microscopy (Nikon ECLIPSE - TE200 with EZ-C1 software, Nikon Instruments, Inc. Melville, New York) at excitation wavelengths of 488 and 543 nm. For image analysis, a maximal projection of each image stack was built using the program Nikon imaging software NIS-Elements AR3.00, SP-4, Hotfix6 (Build, 505) and red and green projections were selected separately to measure their intensity. The resulting red and green intensities were analyzed for their statistical significance. Three independent experiments were carried out for each experimental condition. In each case, the mean optical density of the negative control was subtracted from the readings of all other samples.

Scanning electron microscopy (SEM) observation for the slanted glass coverslip method. For SEM observation biofilms were formed as described in the above biofilm yield assay. Glass slides were gently washed twice with sterilized distilled water. Biofilms on glass coverslips were gently washed twice with sterilized distilled water. Fixation of biofilms on glass slides was performed by the method of Araujo et al. (2003), and fixed biofilms were dehydrated for 10 min in a graded series of cold ethanol/water mixtures (50, 70, 80, 90, 95 and 100% of ethanol). The last dehydration (100% ethanol) was repeated for better sample preparation. With gentle rocking, biofilms on glass slides were treated with 50% ethanol with 50% HMDS (hexamethyldisilazane) for 5 min, and, finally, a 100 min exposure of samples with 100% HMDS. After dehydration, biofilms were air-dried under the hood. After drying, gold-palladium (50%/50%) coatings were applied to the fixed bacterial samples using a 6.2 Hummer Sputter Coater (Anatech USA, Union City, CA). After sputter coating, samples were imaged in five random spots with a SIGMA VP40 Scanning Electron Microscope (2 kV with high vacuum mode).

Extracellular matrix proteins as a substrate for biofilm assay. For ECM studies, six types of coverslips (5 glass coverslips, each with a different extracellular matrix, and 1 plain glass coverslip) were placed on the bottom of 6 well plates. Collagen I, fibronectin, poly-D-lysine/laminin, poly-L-lysine, poly-D-lysine coated coverslips (BD Bioscience, NJ) and plain glass coverslips were used. 200 μ l of *A. baumannii* inoculum was placed on the coverslips and allowed to sit for 5 min for adherence. Following this, 4 ml of nutrient broth was supplied to all wells and plates and incubated under aerobic conditions for 72 h at 37°C. Every 12 h during incubation, the medium containing suspended grown bacterial cells was removed and replaced with the same volume of fresh medium. One group was maintained in a dynamic (100 RPM) condition and another in a static condition. After 72 h, biofilms from both the dynamic and static conditions were used for SEM imaging and crystal violet biofilm quantification assay (method as described in the above biofilm yield assay). Prior to SEM imaging, all samples were smoothly rinsed two times using aseptic distilled water. Fixation of biofilms on immersed ECM for crystal violet quantification studies and SEM imaging was performed as per the above de-

Fig. 1. Crystal violet staining and scanning electron microscopy (SEM) images shows *Acinetobacter baumannii* (BAA-1605) growth characteristics.

A. Glass test tube shows the air-liquid interface biofilm formation. B. 24-well plate shows air-liquid interface biofilm formation. C. 24-well plate shows no biofilm formation on the bottom. D. 24-well plate with glass coverslip shows biofilm formation. E. Glass coverslip with dense biofilm. F. Immersed glass coverslip shows no biofilm formation (SEM, Bar 10 μm). G. Slanted glass coverslip shows dense biofilm formation (SEM, Bar 2 μm).

scribed slanted coverslip method for both the dynamic and static conditions.

Statistical analysis. The means and standard deviations were calculated. One-way (biofilm yield assay) and two-way ANOVA (live/dead staining assays) were used to determine the levels of significance ($p < 0.05$).

Results and Discussions

A knowledge of the factors enhancing *in vitro* *A. baumannii* biofilm growth is lacking. We were initially hindered in antimicrobial testing against *A. baumannii* because of its biofilm growth pattern *in vitro*. This bacterium is a strictly aerobic bacterium that does not form a contiguous cell layer even after extended incubation (Tomaras et al., 2003) and grows only at the air-liquid interface. The strains had weak adhesion patterns to various materials during experimental conditions with the exception of glass tube surfaces (Costa et al., 2006; Egwari and Taiwo, 2004). We used slanted coverslip inserts and six different extracellu-

lar matrixes for the enhancement of biofilm growth and better experimental handling for adherence assay, confocal, and scanning electron microscopy studies.

Biofilm yield assay with, and without, SGC revealed higher levels of biofilm formation for *A. baumannii* strains when grown with SGC inserts. SGC biofilm yield assay showed higher levels and continuous layers of biofilm formation up to 1.7 to 3-fold higher than when carried out without SGC inserts (Figs. 1 and 2). SGC grown *A. baumannii* biofilm stained with a LIVE/DEAD® BacLight™ Bacterial Viability Kit is shown in Figs. 3 and 4. In 72-h confocal micrographs of *A. baumannii* biofilm, fluorescence indicates a thicker biofilm formation. The intensity of the biofilm varied from 17 to 25 million arbitrary units (AU) and the highest biofilm formation was observed with S1 (ATTC BAA-1605).

SEM examination was carried out to check for a possible morphological arrangement in *A. baumannii* during biofilm formation. Cell surfaces were remarkably integrated and regular boundaries were observed and the mar-

gins of cell walls were very clear. Bacterial cells appeared to have very good adherence and cohesiveness behavior, apparently indicating that slanted coverslips provide a healthy environment for biofilm formation. The enlarged insert images of *A. baumannii* biofilms show a clear view of the integration of the biofilm (Fig. 4). Biofilm yield assay results indicate that the use of SGC is a successful methodology for trouble-free handling of enhanced biofilm growth in an air-liquid interface under a static condition. *A. baumannii* has the ability to form a biofilm on a variety of abiotic surfaces such as glass and polystyrene (Jawad et al., 1998; Wendt et al., 1997). Jawad et al. (1998) observed that 39 hospital strains exposed to desiccation on glass coverslips survived up to 27 days; hence, desiccation adaptability occurring more in gram-negative bacterial groups than in gram-positive groups also favors adapt-

ability in *A. baumannii*. Because of this adaptability, biofilms formed on abiotic surfaces are able to extend quorum sensing pathways and pili formation (Chow et al., 2014; Luo et al., 2015; Solano et al., 2014).

Multiple layers within biofilm communities produce strong adhesion and cohesive behavior and are sheathed with extracellular matrix substances of protein, polysaccharides, and other macromolecules (Costerton et al., 1999). Tomaras et al. (2003) reported that *A. baumannii* has a greater attraction to hydrophilic glass surfaces and exhibits dense adherence and cohesive behaviors located at the air-liquid interface. *A. baumannii*, strictly aerobic, does not form dense adherence, cohesive behaviors, and

Fig. 2. *Acinetobacter baumannii* (S1 - BAA-1605; S2 - ATCC-17961; S3 - ATCC-19606 and S4 - ATCC-19003) biofilm yield with and without slanted glass coverslips (SGC) insert.

Fig. 3. Slanted glass coverslips grown *Acinetobacter baumannii* (S1 - BAA-1605; S2 - ATCC-17961; S3 - ATCC-19606 and S4 - ATCC-19003) biofilm quantity measured with BacLight Live/Dead stain.

Fig. 4. Confocal (upper row) and scanning electron (lower row) micrographs of *Acinetobacter baumannii* biofilms.

(S1 - BAA-1605; S2 - ATCC-17961; S3 - ATCC-19606 and S4 - ATCC-19003) formed with slanted glass coverslips. (Bar upper row 50 μ m; lower row 5 μ m).

Fig. 5. Scanning electron micrographs of *A. baumannii* (ATCC BAA-1605) biofilm formation on extracellular matrix and plain glass cover slips (X3000, Bar 10 μm) with magnified insert (X15000, Bar 1 μm).

(A, B - Collagen I coated; C, D - Fibronectin coated; E, F - Poly-D-Lysine/Laminin coated G, H - Poly-L-Lysine coated I, J, - Poly-D-Lysine coated and K, L - Plain glass; A, C, E, G, I and K grown in static condition, B, D, F, H, J, and L grown under dynamic conditions). Arrow marks indicate pili formation in the biofilm. Both sides corresponding crystal violet-stained extracellular matrix slides depicted.

biofilm formation when immersed in plates or tubes even after prolonged incubation, and is able to grow only at the air-liquid interface. Above all, these findings strongly support the slanted coverslips methodology. Biofilm assays (immersed in media without air-liquid interface) with an extracellular matrix achieved thick compact biofilms with

tightly grouped cells of uniform size and morphology which were observed in biofilms grown under dynamic conditions (Figs. 5B, D, F, H, J and L). Collagen I, fibronectin, poly-D-lysine/laminin and poly-D-lysine showed greater biofilm formation than did poly-L-lysine and plain cover-slips. Biofilms grown under static condi-

Fig. 6. Biofilm formation abilities of extracellular matrix and plain glass cover slips by *A. baumannii* (ATCC BAA-1605).

(CL-I - Collagen I coated; FB - Fibronectin coated; PD-LL - Poly-D-Lysine/Laminin coated P-LL - Poly-L-Lysine coated P-DL - Poly-D-Lysine coated and PG - Plain glass as control) statistical significance made comparison with plain glass coverslips bacterial biofilm density (* $P < 0.0001$; ** $P < 0.00001$; *** $P < 0.00000001$).

tions (Figs. 5A, C, E and G) and on plain coverslips (Fig. 1K) were not compact, with very few colonies except in the case of poly-D-lysine (Fig. 5I). Plain glass coverslips showed fewer colonies compared with cell matrix-coated coverslips under both dynamic and static conditions.

Careful inspection of SEM images showed thin fimbrial-like extensions on bacterial surfaces that were firmly anchored to the extracellular matrix (Fig. 5 insert shown with arrow mark). In Figs. 5B, D, F, H, J and L insert bacterial cells seemed to be joined to one another by hair-like (pili) structures. These hairy arrangements were seen in bacterial cells from all groups and likely promote bacterial interaction and biofilm formation. Interestingly, short pili were seen mainly at the interface between the bacterial surface and the cell matrix surface, suggesting their involvement in adherence, cohesive nature, and biofilm formation. Closer views of cells grown under static conditions show bacterial cells with a dying, unhealthy appearance (Figs. 5A, C, E, G, K insert), except in the case of poly-D-lysine (Fig. 5I). Thread-like pili with substrate interaction were not found on plain glass surfaces or on the multilayer bacterial cells sheltered by a film-like structure representing the exopolysaccharide produced by *A. baumannii* (Fig. 5D).

The present study revealed that biofilm grown on extracellular matrixes showed thread-like pili structures, and that these structures had contact with the ECM and were also involved in cell-to-cell interaction. In addition, multilayer bacterial cells formed a thick biofilm sheltered by a film-like structure representing the exo-polysaccharide (Gospodarek et al., 1998). Bacterial exo-polysaccharide plays an important role in adhesion to sister cells and to ECM and forms a suitable environment for biofilm formation. Gospodarek et al. (1998) showed that different thicknesses of hairy-like pili were present in the adher-

ence of *A. baumannii* to human cells and proposed that biofilm formation progression was arbitrated by exopolysaccharides and pili. Pili, flagella, adhesions, membrane proteins, and quorum sensing molecules play important roles in biofilm formation (Starkey et al., 2004). *Acinetobacter* has the capacity to adhere, colonize, and form biofilm on patients' skin and mucosal surfaces (Berlau et al., 1999). A study showed that *Acinetobacter* species could bind to buccal epithelial cells of humans (Vanechoutte et al., 1999). Lee et al. (2006) also portrayed the existence of thin, hairy pili on the surfaces of an *A. baumannii* strain that anchored the bacterium to the membrane surface of human bronchial epithelial cells, and also that the blaPER1 adherence gene expression differed with the adhering surface (Rao et al., 2008). These observations support choosing ECM as a good option for biofilm formation. The absence of pili-like structures on immersed plain coverslips raises the question as to whether the presence of a particular surface triggers the production of pili by *A. baumannii*. Plain glass substrates also showed less biofilm formation compared with ECM substrate and glass adherence (Tomaras et al., 2003; Vidal et al., 1996).

The crystal violet assay described by Djordjevic et al. (2002) for the quantification of biofilm formation can be used to detect biofilm formation on many types of substrata. Crystal violet biofilm quantification results indicated that higher values were observed in biofilms grown under dynamic conditions than under static conditions except in the case of poly-D-lysine coating. Uncoated plain coverslips had lower values compared with ECM-coated coverslips under both dynamic and static conditions. Differences in biofilm quantification were statistically significant ($P < 0.05$) when comparing uncoated plain glass with ECM-coated coverslips, and also when comparing dynamic and static biofilm conditions. Experiments were

performed in triplicate and the means and standard deviations calculated. One-way ANOVA was selected to conclude the level of significance ($p < 0.05$). Four strains of *A. baumannii* were used for this study, but we have exhibited only ATCC BAA-1605 for this paper. SEM observations were supported by crystal violet biofilm quantification (Figs. 5 and 6). Higher values were observed for biofilm grown under dynamic conditions than under static conditions, except for poly-D-lysine coating. Poly-D-lysine may be enhancing the detachment by inhibiting the adherence factor. Uncoated plain coverslips had lower values compared with ECM-coated coverslips under both dynamic and static biofilm-forming conditions.

Current findings support the proposal that fluid movements in the mouth cavity result in biofilm-forming capacity as a function of oxygen availability. Several studies have confirmed that dynamic conditions favor strong biofilm formation compared with static conditions (Biswas and Chaffin, 2005; Hawser et al., 1998). For instance, Hawser et al. (1998) have described the quantitative amplification of bacterial biofilm formation on catheter surfaces as correlated with liquid flow, in contrast with a static environment with no liquid flow. The above finding indicates that environmental nutrients and oxygen enormously support the formation of biofilm layers, particularly the biofilm inner core. The failure of *A. baumannii* to form a biofilm under static environmental conditions may be a result of the mechanical detachment of adhered bacteria from the surface or the collapse of sessile cell metabolism due to a toxic environment resulting from the static conditions. During *A. baumannii* biofilm formation, shear activity likely causes anti-adherence activity in flow chambers, whereas in the static condition only weak shear movements occur. As a result, mechanical anti-adherence activity of sessile cells is probably not the reason why a sessile biofilm does not accumulate. Moreover, in the absence of shear forces (static conditions) sessile *A. baumannii* also does not seem to grow. Tomaras et al. (2003) observed that *A. baumannii* is a strict aerobe which grows only at the air-liquid interface due to oxygen and nutrient requirements. In summary, *A. baumannii* shows higher biofilm formation capacities with different cell matrices, while the prominent measurements of results were directly related to the biofilm formation capacity of *A. baumannii*. For the initial step of biofilm formation, adherence is important and, consequently, strains with a comparatively high capability to adhere to extracellular matrixes are a new method of enhanced biofilm growth for *in vitro* assays.

Conclusions

In conclusion, *A. baumannii* shows higher adhesion, cohesive, and biofilm formation capacities with ECM, and a comparatively high capability of adherence to extracellular matrixes (ECM) and slanted glass coverslips (SGC). This new approach utilized a variety of ECM and SGC. Enhanced biofilm formation was observed in air-liquid interfaces (SGC) and in immersed culture conditions (ECM). These substrates can provide stress-free culture conditions for enhanced biofilm growth for *in vitro* adherence and biofilm assays. Hence, ECM can be used as a

substrate for immersed biofilm formation studies with dynamic conditions and the SGC method can be used for exposed biofilm formation studies under static conditions. This study indicates the possibility to use ECM in biofilm reactors for stress-free culture conditions to enhance biofilm growth.

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