Microbiology (2013), **159**, 2594–2604 **DOI** 10.1099/mic.0.068825-0

Use of a stainless steel washer platform to study *Acinetobacter baumannii* adhesion and biofilm formation on abiotic surfaces

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Acinetobacter baumannii is a frequent cause of hospital-acquired pneumonia, and has recently increased in incidence as the causative agent of severe disease in troops wounded in Afghanistan and Iraq. Clinical approaches are limited since *A. baumannii* strains isolated from patients are extremely resistant to current antimicrobials. *A. baumannii* can survive desiccation and during outbreaks has been recovered from various sites in the patients' environment. To better understand its prevalence in hospital settings, we used a stainless steel washer (SSW) platform to investigate *A. baumannii* biofilm formation on abiotic surfaces. Scanning electron microscopy demonstrated that *A. baumannii* forms strong biofilms on stainless steel surfaces. This platform was combined with a colorimetric 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay to observe the metabolic activity of bacterial cells, and to facilitate the manipulation and comparison of multiple *A. baumannii* clinical strains. A strong correlation between XTT and c.f.u. assays was demonstrated. To complement the cell viability assays, *A. baumannii* biofilm mass was measured by crystal violet staining. Furthermore, the effect of commonly used disinfectants and environmental stressors on *A. baumannii* biofilms and planktonic cells was compared and characterized. Biofilms on SSWs were significantly more resistant than their planktonic counterparts, providing additional evidence that may allow us to understand the high prevalence of this microbe in hospital settings. Our results validate that SSWs are a simple, versatile and innovative method to study *A. baumannii* biofilms *in vitro*.

Received 22 April 2013
Accepted 10 September 2013

INTRODUCTION

Acinetobacter baumannii is a Gram-negative, aerobic, multi-drug-resistant coccobaccillus that is increasing in importance as a potential pathogen in the clinical setting (Fournier *et al.*, 2006). *A. baumannii* is an opportunistic pathogen and a significant cause of nosocomial infections, although it is occasionally associated with community-acquired infections. Whilst *A. baumannii* can be cultured from the skin of healthy individuals, it is more prevalent among patients in the hospital environment (Fournier *et al.*, 2006). One of the main concerns regarding *A. baumannii* is its ability to persist in the hospital environment on various abiotic materials. This allows susceptible patients to come into contact with the organism, often resulting in outbreaks of ventilator-associated pneumonia, meningitis, bacteraemia, urinary tract infections and wound infections (Sheppard *et al.*,

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Abbreviations: SEM, scanning electron microscopy; SSW, stainless steel washer; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide.

2010). These infections are often difficult to treat due to the emergence of multidrug-resistant *A. baumannii* strains (Bonomo & Szabo, 2006; Hujer *et al.*, 2006; Navon-Venezia *et al.*, 2007). These findings indicate that *A. baumannii* is a potential emerging nosocomial threat worldwide.

Whilst there have been studies defining important bacterial characteristics expressed by *A. baumannii*, the mechanisms of virulence and persistence remain largely undefined. The mechanisms that have been described revolve around the organism's outer membrane protein A (OmpA) porin protein (Choi *et al.*, 2005), K1 capsular polysaccharide (Russo *et al.*, 2010), cell wall lipopolysaccharide (Luke *et al.*, 2010), antimicrobial resistance genes (Bonomo & Szabo, 2006; Hujer *et al.*, 2006; Navon-Venezia *et al.*, 2007) and plasmids that carry genes for peroxide resistance (Dorsey *et al.*, 2006). Although little is known about the mechanisms of pathogenesis in *A. baumannii*, the organism's ability to persist in the environment on abiotic surfaces has been a key factor linked to biofilm formation (Choi *et al.*, 2009; de Breij *et al.*, 2010). In *A. baumannii*, the CsuA/BABCDE chaperone–usher assembly system of pili is involved in the initial surface attachment during biofilm formation, with expression of *csuE* being integral in this regard (de Breij *et al.*, 2009). In addition, poly-β-(1,6)-*N*-acetylglucosamine is an extracellular polysaccharide that is thought to function as an intercellular adhesin for the organism within the biofilm (Choi *et al.*, 2009). The *blaPER-1* gene might be associated with increased cell adhesiveness and increased biofilm formation (Lee *et al.*, 2008). However, Rao *et al.* (2008) showed no correlation between *A. baumannii* biofilm formation and *blaPER-1* expression. Finally, the *A. baumannii* biofilm-associated protein (Bap) has been shown to play an important role in biofilm maturation and maintenance (Loehfelm *et al.*, 2008).

As a structural community, a bacterial biofilm is enclosed in a polymeric matrix that protects the organisms against harsh environments, including host and defence mechanisms activated during infection (Donlan, 2002). Biofilm-forming bacteria are also more resistant to antimicrobial stressors, antibiotics and disinfectants. Although limited evidence is available, *A. baumannii* biofilms can survive in dry conditions and the organisms have been recovered during outbreaks from the patients' environment, including bed curtains, furniture and hospital equipment (Dijkshoorn *et al.*, 2007). Terminal cleaning of patients' rooms has been successful in halting outbreaks of *A. baumannii* and emphasizes the role of the hospital environment in being a reservoir for *A. baumannii* (Dijkshoorn *et al.*, 2007). Hence, the objective of the present paper was to develop a flexible approach to study *A. baumannii* biofilms. We used stainless steel washers (SSWs) as abiotic surface to investigate *A. baumannii* biofilm formation and desiccation, thus mimicking the surfaces found in the hospital setting. We combined this platform with the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay and crystal violet staining to facilitate the manipulation of multiple strains and conditions used during the study. Finally, we documented the effect of commonly used disinfectants and environmental stressors on *A. baumannii* biofilm and planktonic cell viability.

METHODS

***A. baumannii*.** The strain *A. baumannii* 0057 was obtained from Mark D. Adams (Cleveland, OH, USA), and used as a reference strain in the present study. This isolate was chosen because of its multiple resistances to antibiotics and the fact that it had been sequenced. *A. baumannii* 0057 strain was isolated in 2004 from a blood culture performed on a soldier at the Walter Reed Army Medical Center (Washington, DC, USA). The *A. baumannii* clinical strains used in this study were isolated from wound cultures at the Montefiore Medical Center. All samples were obtained with written patient consent according to the practices and standards of the institutional review boards at the Albert Einstein College of Medicine and Montefiore Medical Center. In addition, all studies were conducted in accordance with the Declaration of Helsinki Principles. In total, 12 clinical isolates (*A. baumannii* 0248, 0323, 1317, 1422, 1611, 2098, 2231, 2862, 3260, 3659, 7405 and 9149) were included in the study. The antimicrobial susceptibility profile for each clinical isolate tested in this study is shown in Table 1. The strains were stored at −80 °C in brain heart infusion (BD) broth with 40 % glycerol until use. Test organisms were grown in a tryptic soy broth (TSB; MP Biomedicals) overnight at 37 °C using a rotary shaker set at 150 r.p.m. Growth was monitored by measuring the OD_{600} (Bio-Tek).

Biofilm formation. SSWs were used as a substrate to study biofilm formation. Briefly, for each strain, an inoculum of $4–5 \times 10^6$ *A. baumannii* cells was suspended in a 50 ml Erlenmeyer flask with 25 ml of TSB containing 6 mm diameter SSWs. The flasks were then incubated on a rotary shaker set at 150 r.p.m. for 24 h at 37 °C. The SSWs containing *A. baumannii* biofilms were then removed from the flask using flamed-sterilized forceps. To remove non-adhered bacterial cells, the abiotic surface was washed three times with PBS.

Quantification of biofilms. Measurement of biofilm formation and viability was done by c.f.u. determination and assessing the metabolic activity of the attached cells with the XTT (Sigma) reduction assay (Chandra *et al.*, 2008).

(i) **Direct c.f.u. determinations in biofilms.** SSWs were transferred from flasks or wells to a microcentrifuge tube containing 1 ml of PBS and sonicated to detach the cells as described (Merritt *et al.*, 2005). An aliquot of 100 µl of the dissociated cell suspension was transferred to another microcentrifuge tube containing 900 µl of PBS. The suspension was then gently homogenized for 3 min. Twofold serial dilutions of the suspensions were then performed and aliquots of 100 µl from each dilution were plated onto tryptic soy agar (TSA) plates.

(ii) **XTT reduction assay.** SSWs coated with *A. baumannii* biofilms were gently washed with PBS to remove non-adherent bacteria. Each SSW was added to a well of a 96-well plate. Aliquots of 50 µl of XTT salt solution (1 mg ml^{-1} in PBS) and 4 µl of menadione solution (1 mM in acetone; Sigma) were added to each well of a microtitre plate. Microtitre plates were then incubated at 37 °C for 5 h. The electron transport system in the cellular membrane of live bacteria reduces the XTT tetrazolium salt to XTT formazan, resulting in a colorimetric change, which was

Table 1. Antimicrobial susceptibility profile of *A. baumannii* clinical strains tested in this study

Clinical isolate	Antimicrobial drug*												
	Ampicillin	Ampicillin/sulbactam	Cefazolin	Cefoxitin	Ceftazidime	Ceftriaxone	Cefepime	Imipenem	Amikacin	Gentamicin	Tobramycin	Ciprofloxacin	Levofloxacin	Nitrofurantoin	Trimethoprim/sulfamethoxazole
0248	⩾32/R	16/I	⩾64/R	⩾64/R	⩾64/R	⩾64/R	⩾64/R	⩾16/R	16/S	⩾16/R	⩽1/S	⩾4/R	⩾8/R	256/R	⩾320/R
0323	⩾32/R	⩾32/R	⩾64/R	⩾64/R	⩾64/R	⩾64/R	⩾64/R	⩾16/R	⩽2/S	4/S	2/S	⩾4/R	⩾8/R	⩾512/R	⩾320/R
1317	⩾32/R	8/S	⩾64/R	⩾64/R	⩾64/R	⩾64/R	32/R	⩾16/R	8/S	8/I	⩽1/S	⩾4/R	⩾8/R	⩾512/R	⩾320/R
1422	⩾32/R	⩽2/S	⩾64/R	⩾64/R	4/S	16/I	4/S	⩽1/S	⩽2/S	⩽1/S	⩽1/S	⩽0.25/S	⩽0.12/S	⩾512/R	⩾320/R
1611	⩾32/R	8/S	⩾64/R	⩾64/R	⩾64/R	⩾64/R	⩾64/R	⩾16/R	16/S	⩾16/R	⩽1/S	⩾4/R	⩾8/R	⩾512/R	⩾320/R
2098	⩾32/R	⩾32/R	⩾64/R	⩾64/R	4/S	16/I	⩾64/R	⩾16/R	⩽2/S	⩾16/R	⩽1/S	⩾4/R	⩾8/R	⩾512/R	⩾320/R
2231	⩾32/R	16/I	⩾64/R	⩾64/R	16/I	16/I	⩾64/R	⩾16/R	16/S	⩾16/R	⩾16/R	⩽0.25/S	⩽0.12/S	⩾512/R	⩾320/R
2862	⩾32/R	8/S	⩾64/R	⩾64/R	⩾64/R	⩾64/R	⩾64/R	⩾16/R	32/I	8/I	⩾16/R	⩾4/R	4/I	⩾512/R	⩾320/R
3260	⩾32/R	16/I	⩾64/R	⩾64/R	16/I	32/I	32/R	⩾16/R	16/S	⩾16/R	⩾16/R	⩾4/R	⩾8/R	⩾512/R	⩾320/R
3659	⩾32/R	⩾32/R	⩾64/R	⩾64/R	16/I	16/I	⩾64/R	⩾16/R	⩽2/S	⩾16/R	⩾16/R	⩾4/R	⩾8/R	⩾512/R	⩾320/R
7405	⩾32/R	⩾32/R	⩾64/R	⩾64/R	16/I	⩾64/R	⩾64/R	⩾16/R	⩽2/S	⩽1/S	⩽1/S	⩾4/R	4/I	256/R	⩽20/S
9149	⩾32/R	⩾32/R	⩾64/R	⩾64/R	16/I	⩾64/R	⩾64/R	⩾16/R	16/S	⩾16/R	⩾16/R	⩾4/R	⩾8/R	⩾512/R	⩾320/R

*Results are presented as MIC (μg ml^{-1}) and susceptibility interpretation (R, resistant; S, susceptible; I, inconclusive), respectively.

measured in a microtitre reader at 492 nm. Microtitre wells containing clean, autoclaved SSW and only PBS medium without *A. baumannii* bacterial cells were used as negative controls.

For the c.f.u. and XTT assays described above, the cell viability or the percentage of metabolic activity was determined by counting the colonies on TSA plates or by measuring the optical density, respectively. These assays were done to monitor the biofilms exposed to desiccation, disinfectants, heat or UV light relative to unexposed biofilms. All assays were carried out in triplicate.

(**iii**) **Crystal violet assay.** *A. baumannii* biofilm formation on SSWs was measured by crystal violet staining, modifying protocols described previously (Antunes *et al.*, 2011; O'Toole, 2011). SSWs coated with *A. baumannii* biofilms were gently washed with PBS to remove non-adherent bacteria. Each SSW was added to a well of a 96-well plate, air-dried and stained with 0.1 % crystal violet solution for 15 min. SSWs were rinsed three times with distilled water (dH_2O), shaken vigorously using forceps to remove all excess dye, air-dried and transferred to a clean 96-well plate. Then, a suspension of 30 % acetic acid in dH_2O was added to each well containing SSWs to solubilize the crystal violet and the plate was incubated at room temperature for 15 min. Finally, SSWs were removed from each well and solubilized crystal violet was measured in a microtitre reader at 550 nm using 30 % acetic acid in dH_2O as negative control.

Differences in cell viability (c.f.u.), metabolic activity (XTT reduction) and crystal violet measurements among *A. baumannii* strains were used as criterion to determine whether a strain formed strong, moderate or weak biofilms on SSWs.

Correlation between XTT and c.f.u. assays in measuring cell viability on biofilms. *A. baumannii* biofilms were formed over a series of time intervals (2, 4, 8, 24 and 48 h) on SSWs. The metabolic activity and bacterial mass were measured by XTT reduction and c.f.u. counts, respectively. The correlation between the XTT reduction and c.f.u. assay results in quantifying bacterial biofilm formation was then evaluated.

Susceptibility of *A. baumannii* planktonic cell and biofilms to desiccation. The resistance of *A. baumannii* biofilms and planktonic cells to desiccation was measured, slightly modifying protocols published previously (Antunes *et al.*, 2011; Jawad *et al.*, 1998). For planktonic cells, bacteria were grown in TSB for 24 h and a 10^7 c.f.u. suspension was deposited onto sterile SSWs. Biofilms were allowed to grow on SSWs for 24 h, as described earlier. SSWs were then removed from the organism culture broths and extensively washed with PBS to remove non-adhered cells. For both phenotypes, SSWs were transferred onto an uncovered Petri dish in an airtight transparent plastic box. The relative humidity inside the plastic boxes was maintained at 31 % by the presence of a saturated salt solution of $CaCl_2$ in an open 5 ml beaker (Jawad *et al.*, 1998). At regular intervals (days 7, 14, 21, 28, 35, 42, 49, 56 and 63), SSWs were transferred to a microcentrifuge tube containing 2 ml of PBS and sonicated for ~1 min to detach the adhered cells. Serial dilutions of the cell suspensions were performed and quantified by the c.f.u. assay described above. Biofilm and planktonic cell metabolic activity was also quantified by the XTT reduction assay. The capability of mature *A. baumannii* biofilms or planktonic cells to survive desiccation was determined by comparing the metabolic activities and number of c.f.u. at each interval time.

Susceptibility of *A. baumannii* biofilms to disinfectants.

(**i**) **Concentration.** To evaluate the susceptibility of the *A. baumannii* biofilms to different disinfectants at varying

concentrations, mature biofilms were grown on SSWs and were then transferred to 1 ml PBS containing ethanol (EtOH), hydrogen peroxide (H_2O_2), alkyl dimethyl ammonium chloride (Lysol) or hypochlorite (NaOCl) (0, 3.12, 6.25, 12.5, 25, 50 or 100 %). The biofilms were vortexed to ensure uniform distribution of the disinfectant on the SSW surface. The SSWs were then incubated at 37 °C for 15 min and biofilm cell viability was quantified by c.f.u. assays. The results obtained were then compared with those of the untreated biofilms, which served as controls.

(ii) **Time.** The effect of exposure time on the susceptibility of *A. baumannii* to different disinfectants was evaluated. SSWs were exposed to MICs of the disinfectants (25 % EtOH, 6.25 % H_2O_2, 6.25 % Lysol or 3.125 % NaOCl), chosen based on the results obtained in the concentration assay. The MIC of disinfectants was determined as the minimum concentration of disinfectants that kills 50 % of the bacterial cells. Samples were processed as described above for different time points (1, 5, 10 and 20 min).

For both antimicrobial susceptibility assays, planktonic cells were also tested to compare their resistance levels with biofilms.

Susceptibility of *A. baumannii* planktonic cells and biofilms to environmental stress.

(i) **Exposure of *A. baumannii* planktonic cells and biofilms to heat.** SSWs containing biofilms by *A. baumannii* or a suspension of *A. baumannii* planktonic cells were transferred to 2 ml microcentrifuge tubes containing 2 ml PBS and then placed in a water bath, set at varying temperatures (37, 39, 41, 43, 45, 47 and 49 °C). After 30 min of exposure at each temperature, the bacterial viability of either planktonic cells or biofilms was measured as described using c.f.u. assays.

(ii) **Effect of UV light irradiation on *A. baumannii* planktonic cells and mature biofilms.** The effects of irradiation by UV light on biofilms formed by *A. baumannii* or planktonic cells were determined by exposing bacterial cells to various doses (100, 200, 300 and 400 mJ cm^{-2}) of UV light (254 nm) generated by a GS Gene Linker UV chamber (Bio-Rad) (McKinney & Pruden, 2012). The viability of planktonic cells or biofilms post-irradiation with UV light was measured by c.f.u. assays and compared with results obtained with non-irradiated cells.

Scanning electron microscopy (SEM) of *A. baumannii* 0057 biofilms. *A. baumannii* 0057 biofilms were grown on SSWs as described above. SSWs with biofilms were then washed three times with PBS, and transferred to a 2 ml microcentrifuge tube containing 2.5 % glutaraldehyde plus 4 % formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, incubated for 2 h at room temperature and post-fixed in 1 % osmium tetroxide. The samples were serially dehydrated in ethanol, fixed in a critical-point dryer (Samdri-790; Tousimis), positioned with a double-sided carbon tape in aluminium stubs and coated with gold in a Balzers gold sputtering system (Bal-Tec). Samples were then viewed with a Quanta 250 (FEI) scanning electron microscope, operating at 20 KV. To quantify the number of bacterial cells on biofilms on various areas (surface, inner, and outer edges) of the SSW, 10 fields (3.6×10^3 μm^2; fields of 60 $\mu m \times 60$ μm were used) for each of those areas were selected randomly. Two separate sets of cultures were prepared for this process.

Statistical analysis. All data were subjected to statistical analysis using PRISM 5.0 (GraphPad). *P*-values were calculated by Student's *t*-test; $P<0.05$ was considered significant.

RESULTS

Kinetics of *A. baumannii* adhesion and biofilm formation

The kinetics of adhesion and biofilm formation by *A. baumannii* 0057 on SSWs was studied using both c.f.u. and colorimetric XTT reduction assays. The cellular mass of the biofilm was found to increase over time, suggesting that the cell number increases per SSW surface (Fig. 1a).

We investigated the correlation between XTT reduction and c.f.u. assays in monitoring *A. baumannii* 0057 biofilm formation (Fig. 1b). A strong correlation was found between the results of the two assays performed ($R^2=0.945$, $P=0.006$). XTT activity was found to have an exponential association with c.f.u. counts. Therefore, XTT colorimetric intensity correlated with increased bacterial mass in the biofilm.

We then used the established protocol to compare the abilities of *A. baumannii* 0057 and 12 clinical strains to form biofilms using the XTT reduction assay after 24 h incubation (Fig. 1c). *A. baumannii* strains 0057, 1422, 1611, 2098, 3659 and 7405 produced extensive biofilms. *A. baumannii* strains 0248 and 2231 were less effective in producing a biofilm. *A. baumannii* isolates 0323, 1317, 2862, 3260 and 9149 were unable to form a biofilm on the stainless steel surface of the SSWs.

To confirm the results obtained using the XTT reduction assay, we utilized the direct bacteria enumeration protocol to quantify biofilm formation by *A. baumannii* strains on the stainless steel surface of the SSWs (Fig. 1d). Most of the strains showed similar biofilm formation capacity. *A. baumannii* strains 0057, 1422, 1611, 3659 and 7405 produced strong biofilms. *A. baumannii* strains 0248, 2098 and 2231 formed moderate biofilms. *A. baumannii* isolates 0323, 1317, 2862, 3260 and 9149 did not produce a biofilm.

Quantification of biofilm only as cell viability (c.f.u.) or activity (XTT reduction assay) provides a partial view of the actual biofilm quantity, as the extracellular matrix largely exceeds, in terms of mass, the cellular component. Hence, biofilm formation on SSWs was compared amongst the different *A. baumannii* strains using the crystal violet method, which stains both cellular and matrix component of biofilms (Fig. 1e). Similar to the c.f.u. and XTT assays, *A. baumannii* strains 0057, 1422 (Fig. 1f), 1611, 2098, 3659 and 7405 produced strong biofilms. *A. baumannii* strains 0248 and 2231 (Fig. 1f) formed moderate biofilms, whereas *A. baumannii* isolates 0323, 1317, 2862 (Fig. 1f), 3260 and 9149 were unable to form biofilms.

Based on the results obtained in c.f.u., XTT reduction and crystal violet quantification, we chose strains 0057, 0248, 1422, 1611, 2098, 2231, 3659 and 7405 to perform desiccation, disinfectant and environmental stress assays.

Visualization of *A. baumannii* 0057 biofilms

Adhesion and biofilm formation by *A. baumannii* 0057 on SSWs was monitored by SEM (Fig. 2). Mature 24 h *A.*

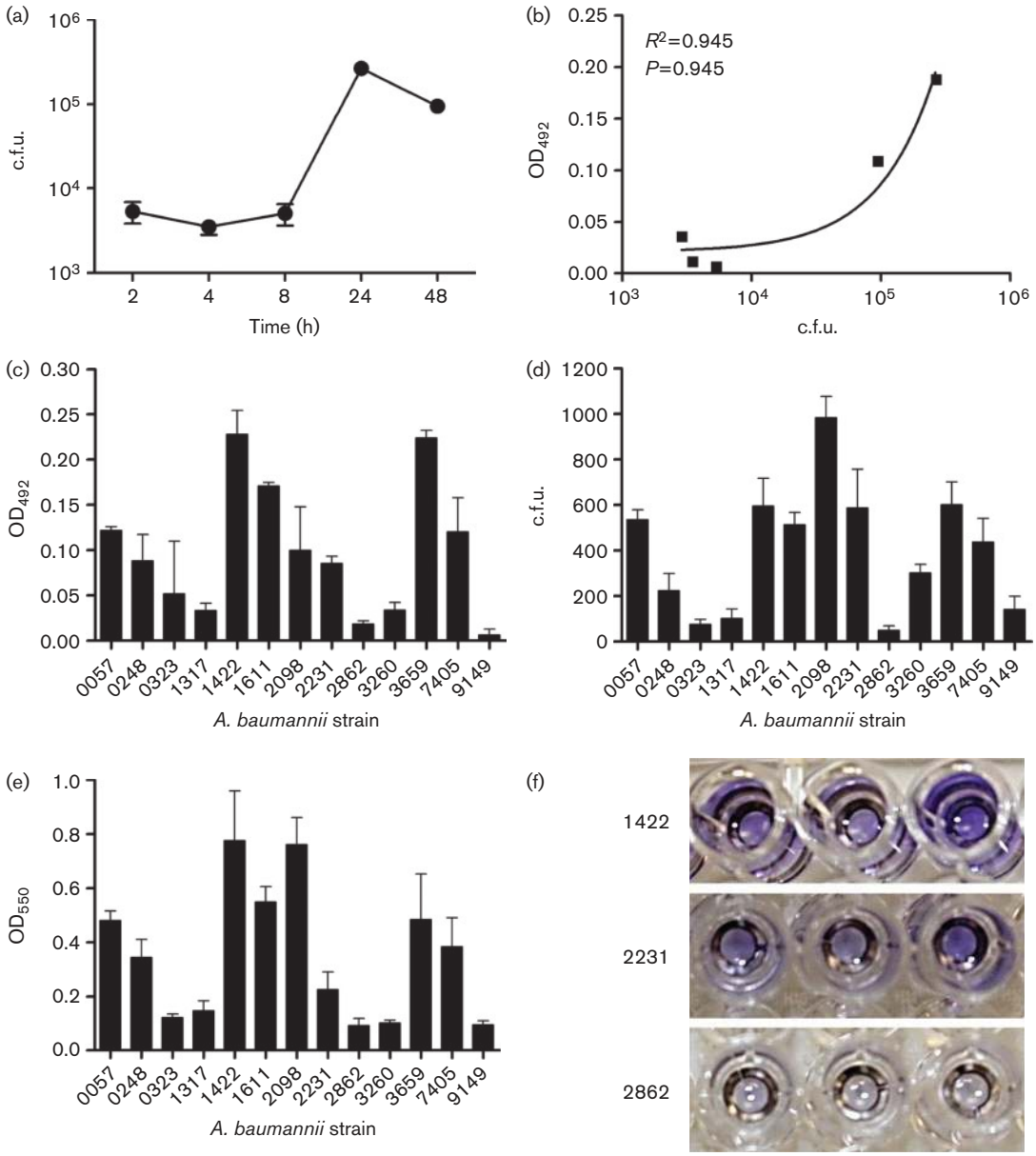

Fig. 1. *A. baumannii* forms a biofilm on SSWs. (a) Kinetics of *A. baumannii* 0057 strain biofilm formation was determined by c.f.u. Each point represents the mean ± SD of three measurements. (b) Correlation between XTT reduction and c.f.u. assays for monitoring *A. baumannii* biofilm formation. R^2 and *P*-values for each regression are also indicated. (c–e) Biofilm formation on SSWs by *A. baumannii* clinical isolates. Clinical *A. baumannii* strain (n=13) biofilm formation was determined by (c) XTT reduction, (d) c.f.u., and (e) crystal violet assays after 24 h. Bars and error bars: mean + SD of six measurements (c, e) and three plates (d) per strain. (f) Top-down images of strong (1422), moderate (2231) and weak (2862) *A. baumannii* biofilms on SSWs after crystal violet solubilization with 30 % acetic acid in dH_2O. For (a–f), results are representative of two experiments.

baumannii 0057 biofilms that adhered to the solid surface (Fig. 2a, b; white squares) were found to consist of a dense group of cells connected internally by extracellular material (Fig. 2c). At the inner edge of the SSW (white arrows), we observed *A. baumannii* 0057 cells interacting with each other (Fig. 2d–f) enmeshed in extracellular material (Fig. 2f).

SEM images were also quantified to assess the number of cells in biofilms attached on the different sections of the SSW. The results revealed that significantly more bacteria attached on the flat surface of SSWs at 48 h (~11×10^3 μm^2; P=0.0017) when compared with 24 h biofilms (~7×10^3 μm^2; Table 2). There were no differences in the numbers of *A. baumannii* cells adhered to the inner or outer edges of the SSWs after 24 or 48 h. Thus, the adherence of *A. baumannii* cells to stainless steel substrate relative to time (24 or 48 h) was inner edge>outer edge>flat surface.

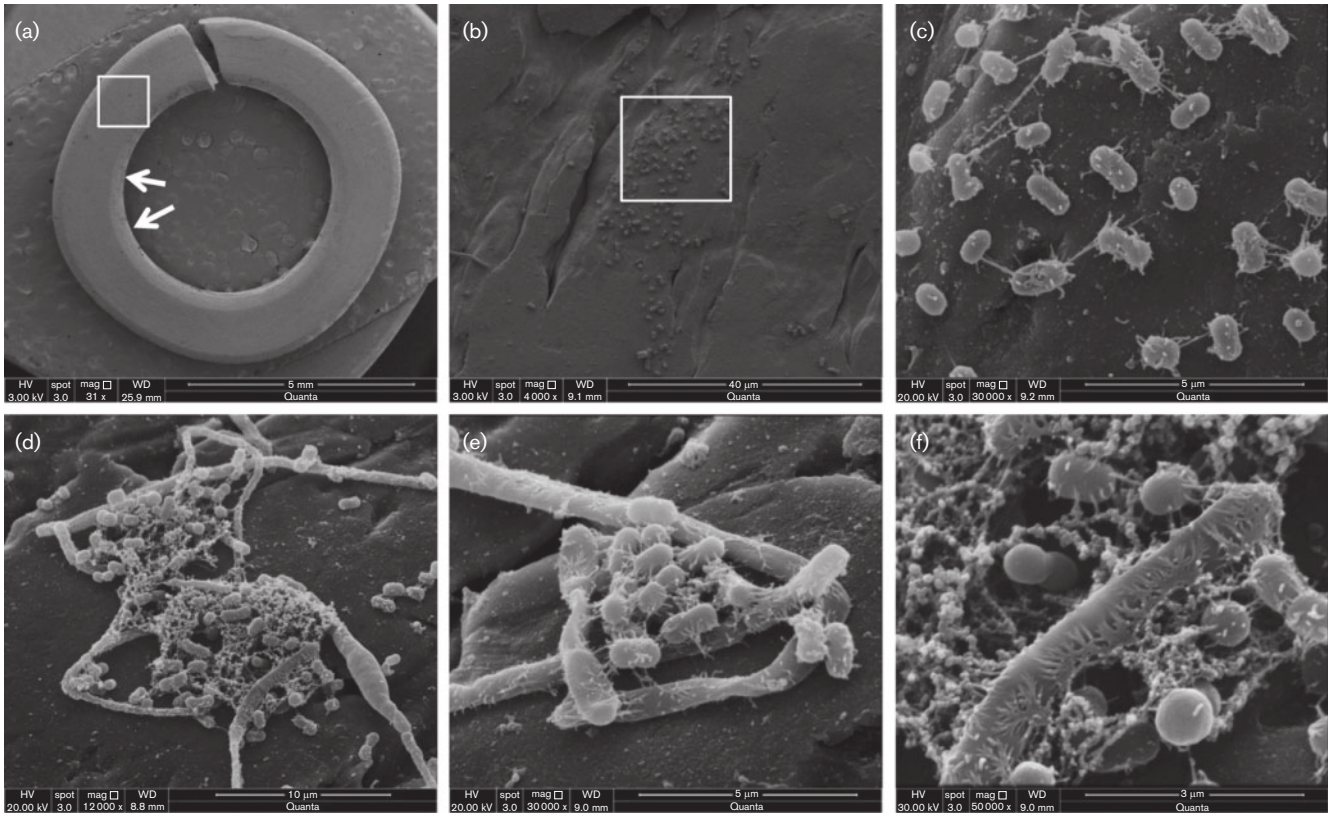

Fig. 2. SEM images of mature *A. baumannii* biofilms (24 h: a–f) formed on SSWs revealed that bacterial cells adhere by way of pili and are surrounded by moderate amounts of extracellular material. White squares in (a) and (b) delineate the magnified region on the next picture. Arrows in (a) denote the edge of the washer.

A. baumannii biofilms are resistant to desiccation

A. baumannii is an important nosocomial pathogen that can survive desiccation for prolonged periods (Antunes *et al.*, 2011; Espinal *et al.*, 2012; Roca *et al.*, 2012). Hence, we evaluated and compared the effects of desiccation on viability of planktonic cells and biofilms by *A. baumannii* strains over a 63 day period using the c.f.u. assay (Fig. 3a). Biofilms were significantly more resistant than planktonic cells by day 14 ($P<0.001$). By day 35, only four out of eight (50 %) cells of planktonic tested strains were viable. Similarly, the planktonic phenotype for all tested strains was dead by day 49. By days 35 and 49, there were means of 2- and 4-log decreases ($P<0.0001$; compared with day 0) in *A. baumannii* cells within biofilms, respectively, in c.f.u. On day 63, at least 1 c.f.u. of the organism within biofilms was detected on desiccated SSWs for six out of eight (75 %) tested strains. Notably, the c.f.u. results were validated by XTT reduction assays (data not shown). These results suggest that the ability of *A. baumannii* to survive in the hospital environment for prolonged periods may well be in part due to its resistance to desiccation.

Table 2. Quantification of *A. baumannii* cells on biofilms visualized by SEM in different locations of SSWs

Area of steel washer	24 h	48 h	*P* (24 versus 48 h)
Flat surface	7 ± 2	11 ± 1*	0.0017*
Inner edge	40 ± 13	40 ± 11	0.9846
Outer edge	22 ± 11	35 ± 6	0.31

Values are expressed as mean (number of bacteria/10^3 μm^2) $\pm$ SEM.
*$P\leqslant0.05$.

SEM analysis of a 7 day desiccated SSW revealed large numbers of *A. baumannii* 0057 bacterial cells adhered to the solid surface encased in vast amounts of extracellular material. *A. baumannii* 0057 biofilms were found to comprise a dense network of bacterial cells which were firmly attached to the solid surface (Fig. 3b–d). Extracellular material was observed surrounding the bacterial cells (Fig. 3d). SEM images of 7 day desiccated *A. baumannii* biofilms were also quantified to evaluate the number of cells adhered on several areas of the SSWs. The adherence of *A. baumannii* cells to stainless steel substrate relative to time was inner edge>flat surface>outer edge (data not shown).

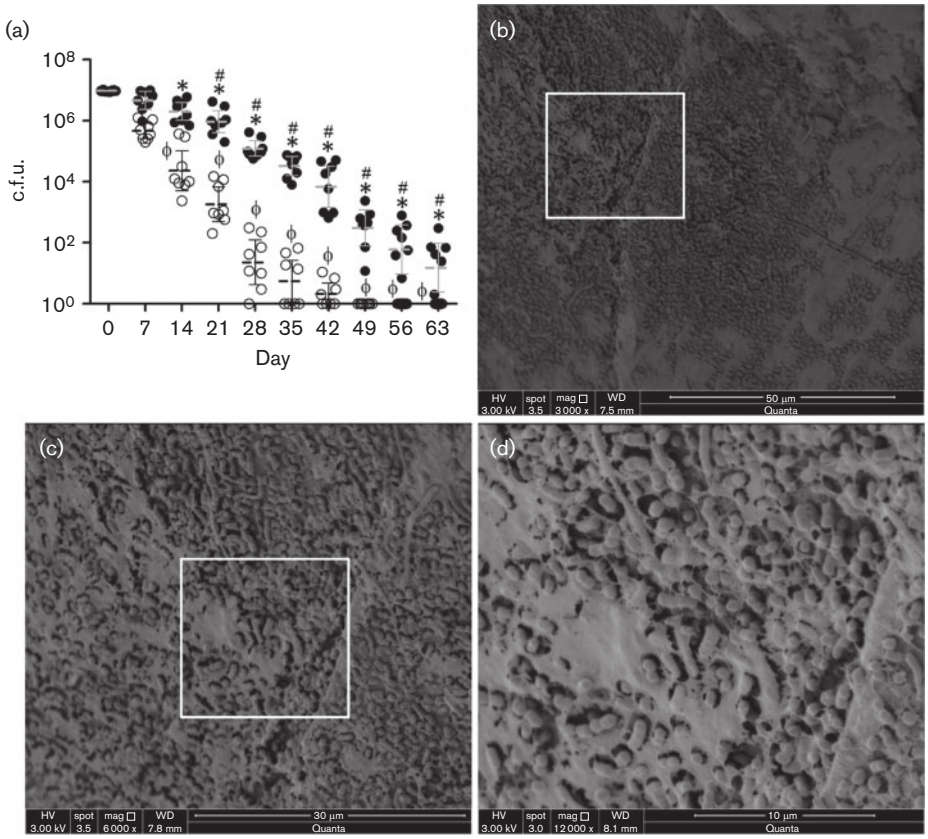

Fig. 3. *A. baumannii* biofilms are more resistant than planktonic cells to desiccation. (a) Survival of *A. baumannii* planktonic cells and biofilms on a dry surface. Each circle (black, biofilms; white, planktonic cells) represents an *A. baumannii* strain ($n=8$) after the mean of three measurements per strain was calculated; error bars: SD for all strains in each experimental group. $P<0.0001$, biofilms (#) or planktonic cells (ϕ) compared with unexposed cells of their phenotype; *$P<0.01$, biofilms compared with planktonic cells. Results are representative of two experiments. (b–f) On day 7, SEM was performed to show the extensive number of *A. baumannii* 0057 cells attached to SSWs and which were surrounded by vast amounts of extracellular material. White squares in (b) and (c) delineate the magnified region in the subsequent panel.

Effect of disinfectants on mature *A. baumannii* biofilms

The efficacy of a disinfectant is dependent on the concentration used and the contact time of exposure. The antibacterial effect of standard disinfectants on biofilm and planktonic cells of *A. baumannii* clinical strains was accessed. Cellular mass was measured by the c.f.u. assay (Table 3). *A. baumannii* biofilms and planktonic cells were found to be similarly and most susceptible to 3.125 % NaOCl with all strains of both phenotypes 100 % susceptible. H_2O_2 and Lysol reduced the c.f.u. of biofilms by ~3–4 logs at concentrations of 6.25 % with 62.5 (H_2O_2) and 37.5 % (Lysol) of strains susceptible to each disinfectant. EtOH was found to be the least effective disinfectant against biofilms, affecting only 25 % of *A. baumannii* strains tested. Hence, the effectiveness of disinfectants relative to concentration was NaOCl>H_2O_2>Lysol>EtOH on biofilms. In contrast, planktonic cells of *A. baumannii* clinical strains displayed 100 % susceptibility to 3.125 % of either Lysol or H_2O_2 and $\geqslant$25 % of EtOH.

We also evaluated the efficacy of disinfectants against *A. baumannii* biofilms and planktonic cells according with their contact time of exposure (0, 1, 5, 10 and 20 min). Similar to the findings obtained with the concentration assay, NaOCl was found to be the most effective disinfectant against *A. baumannii* strains with bacterial cells within biofilms being killed rapidly after 1 min of exposure (Table 3). EtOH reduced the biofilm c.f.u. by ~3–4 logs after 5 min of exposure. H_2O_2 and Lysol were unsuccessful in killing organisms within biofilms even after 20 min of exposure. Thus, the effectiveness of disinfectants relative to time was NaOCl>EtOH>H_2O_2=Lysol on biofilms. Conversely, the viability of *A. baumannii* planktonic cells was completely reduced after 1 min exposure to 3.125 % Lysol, H_2O_2 or NaOCl. Furthermore, EtOH (25 %) was the slowest acting disinfectant, killing *A. baumannii* planktonic cells completely after 5 min of exposure.

Table 3. Effects of four commonly used disinfectants on mature *A. baumannii* biofilms and planktonic cells as a function of concentration and time

Disinfectant	Biofilms				Planktonic cells			
	$n=8$	Concentration (%)	% S*	Time (min)	$n=8$	Concentration (%)	% S*	Time (min)
Alkyl dimethyl ammonium chloride (Lysol)		$\geqslant$6.25–100	37.5	>20		$\geqslant$3.125	100	$\geqslant$1
Ethanol (EtOH)		$\geqslant$25–100	25	$\geqslant$5		$\geqslant$25–50	100	$\geqslant$5
Hydrogen peroxide (H_2O_2)		$\geqslant$6.25–12.5	62.5	>20		$\geqslant$3.125	100	$\geqslant$1
Hypochlorite (NaOCl)		$\geqslant$3.125	100	$\geqslant$1		$\geqslant$3.125	100	$\geqslant$1

*Percentage of isolates susceptible to disinfectant.

Notably, measurement of metabolic activity by the XTT assay gave no results on the biofilm susceptibility assays as the chemical components of the disinfectants used reacted with XTT tetrazolium salt and caused rusting of SSWs. This resulted in interference when measuring the optical density. Hence, the XTT assay was not useful in measuring the effect of disinfectants on mature biofilms by *A. baumannii*.

Susceptibility of *A. baumannii* biofilms to environmental stress

The susceptibility of bacterial planktonic cells or biofilms to harsh environmental condition, such as exposure to high temperatures and UV light, was investigated (Fig. 4). The c.f.u. killing assays were utilized to quantify microbial cellular mass. Biofilms containing *A. baumannii* were significantly more resistant ($P<0.01$) than planktonic cells after 30 min exposure to thermal stress $\geqslant$39 °C (Fig. 4a). Additionally, only biofilms of two out of eight (25 %) tested strains were susceptible to complete thermal eradication after exposure to 49 °C, whereas 100 % of planktonic cells were vulnerable to similar stress. Moreover, biofilms and planktonic cells were reduced significantly in viability ($P<0.0001$; heat-treated compared with control) after 30 min exposure to thermal stress $\geqslant$41 and 39 °C, respectively (Fig. 4a).

Likewise, mature biofilms of clinical strains grown on SSWs and planktonic cells were exposed to UV irradiation, and their bacterial mass in c.f.u. was on average reduced by 2 and 4 logs ($P<0.0001$; UV irradiated compared with control) after exposure to 300 mJ cm^{-2} when compared with their respective unexposed cells (Fig. 4b). Furthermore, *A. baumannii* planktonic cells were significantly more vulnerable ($P<0.01$) than biofilms after exposure to UV irradiation doses $\geqslant$100 mJ cm^{-2} (Fig. 4b). However, although both *A. baumannii* biofilms or planktonic cells were significantly affected by UV stress, complete eradication of either phenotype was not observed.

DISCUSSION

A. baumannii is an important opportunistic pathogen, which has the ability to colonize patients and to persist in the hospital environment, thus posing a significant threat to patients as a nosocomial agent (Dijkshoorn *et al.*, 2007). *A. baumannii* can survive on nutrient-limited surfaces for several days, and is able to resist desiccation and disinfection (Jawad *et al.*, 1998; Wendt *et al.*, 1997). Previous studies suggested that *A. baumannii*'s ability to persist in these conditions is related to its capacity to form biofilms (Gaddy & Actis, 2009). Therefore, in the present paper we used SSWs to investigate *A. baumannii* adhesion and biofilm formation, and its resistance to desiccation on abiotic surfaces, mimicking those found in the hospital setting with the purpose of understanding the ability of clinical isolates to persist in clinical environments and cause disease.

Attachment and biofilm formation on hydrophilic surfaces (e.g. SSWs) by clinical isolates of *A. baumannii* is a property associated with the capacity of this microbe to survive in hospital environments and medical devices, and subsequently cause infections in immune-compromised patients. Using three different methods (c.f.u., XTT reduction and crystal violet assays), we found that eight clinical strains formed strong biofilms on SSWs, whereas five strains formed moderate-to-weak biofilms on SSWs. There is a positive relationship between the degree of bacterial hydrophobicity and adhesion to abiotic surfaces (Costa *et al.*, 2006). In this regard, a recent study has shown that strains of *A. baumannii* with high hydrophobicity index are likely to form strong biofilms under static and dynamic conditions (Pour *et al.*, 2011). Moreover, the production of lectins and extrapolymeric matrix by clinical strains are other important elements in adhesion and pathogenesis (Pour *et al.*, 2011). Together, these factors are reasonable indicators of the ability of biofilm-former strains to persist successfully on medical surfaces (Bergogne-Bérézin *et al.*, 1993).

A. baumannii cells grown on SSWs strongly adhered to a steel support. In other micro-organisms this adherence mechanism is associated with external features on the cell surface, such as fimbriae, flagella or capsules (Bullitt & Makowski, 1995; Martinez & Casadevall, 2005; Rosenberg *et al.*, 1982). SEM analysis showed *A. baumannii* cells

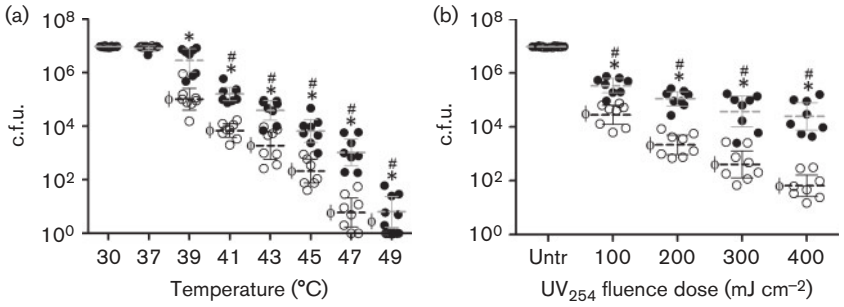

Fig. 4. *A. baumannii* biofilms are more resistant than planktonic cells to environmental stress. (a) *A. baumannii* planktonic cells and biofilms were exposed to 37, 39, 41, 43, 45, 47 and 49 °C for 30 min, and c.f.u. counts were compared between both phenotypes and to those of their phenotype unexposed to heat as a function of time. (b) *A. baumannii* planktonic cells and biofilms were exposed to UV irradiation, and c.f.u. counts were compared between both phenotypes and with those of their phenotype unexposed to UV light as a function of time. For each graph, each circle (black, biofilms; white, planktonic cells) represents an *A. baumannii* strain ($n=8$) after the mean of three measurements per strain was calculated; error bars: SD for all strains in each experimental group. $P<0.0001$, biofilms (#) or planktonic cells (ϕ) compared with unexposed cells of their phenotype; *$P<0.01$, biofilms compared with planktonic cells. Results are representative of two experiments.

linked to each other with extracellular appendages that resemble fimbriae or pili. Previous studies have shown that pili production is essential for biofilm formation by *A. baumannii* (Tomaras *et al.*, 2003, 2008) and may provide the organism with an advantage in the attachment process. It has been found that inactivation of the *csuE* gene results in the abolition of pili, which in turn inhibits cell attachment and biofilm formation (Tomaras *et al.*, 2003). The proximity of bacterial cells and biofilm formation may present a formidable environment for the establishment of nutrient gradients, genetic exchange and quorum sensing. Although mainly single bacterial cells were observed by SEM, *A. baumannii* biofilms were found to consist of dense arrangements of bacterial cells embedded within extracellular material. This structural organization may provide the organism with a sheltered niche against environmental predators, immune cells, shear forces and therapeutic agents.

Adhesion differences to various locations on SSWs by *A. baumannii* cells were observed with a greater number of bacteria attached to the edges than to the flat surface. Previous work has shown that the coarseness found on the edges of stainless steel considerably enhances bacterial cell adhesion and biofilm formation compared with flat or polished surfaces (Arnold & Bailey, 2000). Our findings suggest that nosocomial infections might be reduced if bacterial deposition on medical surfaces is minimized by increasing the use of polished materials that are resistant to microbial colonization. In addition, it is imperative that medical manufacturers, in addition to gaining knowledge about surface morphology to design appropriate materials for the reduction of microbial contamination during medical procedures, acquire an understanding of the process of bacterial attachment and biofilm biology.

Previous studies have already shown that *A. baumannii* is more resistant to desiccation than other *Acinetobacter* species (Jawad *et al.*, 1998). This factor allows the organism to survive for long periods of time on abiotic surfaces and may in part explain the prevalence of this bacterium in the hospital setting. Our findings indicated clearly that *A. baumannii* adhered to SSWs, formed extensive biofilms, and, when desiccated, the majority of the isolates were able to survive between 49 and 63 days, as opposed to their planktonic counterparts that survived 28–42 days. SEM analysis of biofilms showed dehydrated *A. baumannii* cells attached strongly to the solid surface covered with vast amounts of extracellular material. We demonstrated that massive production of this extracellular material is required for both mechanical stability and for longer survival rates during periods of desiccation. Production of extracellular material may also contribute to the organism's resistance to disinfectants and antimicrobials.

In the present paper, we compared the XTT assay to the biofilm c.f.u. quantification method (Merritt *et al.*, 2005) for evaluating biofilm development in *A. baumannii*. Traditionally, c.f.u. determination was used to measure cell viability, although the method is laborious and is wrought with difficulties in disrupting cell aggregates without affecting viability. The XTT reduction assay, which is based on metabolic activity rather than viability, has been used extensively for biofilm quantification in fungi (Chandra *et al.*, 2008; Martinez & Casadevall, 2007; Ramage *et al.*, 2001). The XTT reduction assay is a colorimetric method that quantifies the number of living cells in a biofilm. Our data demonstrated that the c.f.u. assay positively correlates with the XTT readings, suggesting that both methods can be used reliably for quantification of *A. baumannii* biofilm viability. Furthermore, we used the crystal violet method (Antunes *et al.*, 2011; O'Toole, 2011) to stain the biofilms on SSWs – this assay complemented and supported the results obtained by the cell viability methods. The advantage of the XTT assay,

when compared with the c.f.u. method, is that in the former method multiple strains can be manipulated in a 96-well plate format. However, the XTT assay was not a useful tool to study the effects of disinfectants. The chemically active component of the disinfectants reacted in the presence of XTT, causing rusting of SSWs. The false coloration, which was due to the rusted surface, interfered with the measurement of optical density. The c.f.u. killing assays, however, strongly demonstrate resistance of *A. baumannii* biofilms towards disinfectants.

The ability to form a biofilm provides a bacterium with a survival advantage in the natural environment over planktonic organisms, e.g. adhering at a location where growth is favourable, protecting itself from desiccation or predation, and resisting the effects of biocides and detergents. *A. baumannii* is generally found in hospital settings, despite its exposure to many environmental factors. Although our findings showed that there was a significant decrease in *A. baumannii* biofilm c.f.u. after exposure to heat and UV irradiation, the bacterial population was not completely eradicated. This finding suggests that biofilm establishment may protect *A. baumannii* from environmental shifts, which is facilitated by cell-to-cell interactions, with the extracellular material acting as a shield against stress conditions.

Resistance to disinfection enables *A. baumannii* to survive in hospital environments for long periods of time. More importantly, biofilm formation by this organism helps its persistence. We exposed *A. baumannii* biofilms to disinfectants regularly used in healthcare facilities. We found that NaOCl, the active reagent in household bleach, effectively killed *A. baumannii* biofilms. However, although the *A. baumannii* cells within the biofilms were reduced significantly in number, the organism was not completely killed after treatment with EtOH, H_2O_2 or Lysol. In addition to biofilm formation, efflux pump mechanisms present in *A. baumannii* have been shown to contribute to the organism's survival when exposed to disinfectants and antimicrobial agents (Rajamohan *et al.*, 2010). Planktonic cells were significantly susceptible to disinfectants.

In summary, our results show that adhesion to solid surfaces and biofilm formation may provide *A. baumannii* with an advantage and ideal niche for its extended prevalence in hospital settings and in various hostile environments. This phenotype may enhance the ability of the bacterium to resist environmental stress, disinfection and antimicrobial therapy. In addition, the organism's resistance to desiccation may facilitate its colonization and persistence on hospital environmental surfaces, thereby increasing the probability of causing nosocomial infections and outbreaks either by direct contact or horizontal transmission. Furthermore, our study also demonstrated the use of SSWs as a tool to study *A. baumannii* bacterial biofilms along with all of the other methods (c.f.u. assay, XTT assay, crystal violet staining and SEM).

ACKNOWLEDGEMENTS

L. R. M. gratefully acknowledges support from the NIH-NIAID (grant 5K22A1087817-02) and LIU-Post Faculty Committee Research Awards. S. J. O.-J. gratefully acknowledges support from Benjamin Cummings/MACUB Student Research and LIU-Post Faculty Research Committee Undergraduate Research Awards. The authors also thank Rachel Rachid for the excellent work on SEM analysis. Finally, we thank Jade M. Greco and Kimberly Vero for their assistance in the experiments for the revised version of the paper. The authors state no conflict of interest.

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Edited by: D. Hood