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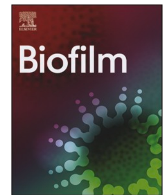

A comprehensive comparison of biofilm formation and capsule production for bacterial survival on hospital surfaces

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A R T I C L E I N F O

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A B S T R A C T

Biofilm formation and capsule production are known microbial strategies used by bacterial pathogens to survive adverse conditions in the hospital environment. The relative importance of these strategies individually is unexplored. This project aims to compare the contributory roles of biofilm formation and capsule production in bacterial survival on hospital surfaces.

Representative strains of bacterial species often causing hospital-acquired infections were selected, including *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. The importance of biofilm formation and capsule production on bacterial survival was evaluated by comparing capsule-positive wild-type and capsule-deficient mutant strains, and biofilm and planktonic growth modes respectively, against three adverse hospital conditions, including desiccation, benzalkonium chloride disinfection and ultraviolet (UV) radiation. Bacterial survival was quantitatively assessed using colony-forming unit (CFU) enumeration and the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay and qualitatively by scanning electron microscopy (SEM). Correlations between capsule production and biofilm formation were further investigated.

Biofilm formation contributed significantly to bacterial survival on hospital surface simulators, mediating high resistance to desiccation, benzalkonium chloride disinfection and UV radiation. The role of capsule production was minor and species-specific; encapsulated *A. baumannii* but not *K. pneumoniae* cells demonstrated slightly increased resistance to desiccation, and neither showed enhanced resistance to benzalkonium chloride. Interestingly, capsule production sensitized *K. pneumoniae* and *A. baumannii* to UV radiation. The loss of capsule in *K. pneumoniae* and *A. baumannii* enhanced biofilm formation, possibly by increasing cell surface hydrophobicity.

In summary, this study confirms the crucial role of biofilm formation in bacterial survival on hospital surfaces. Conversely, encapsulation plays a relatively minor role and may even negatively impact bacterial biofilm formation and hospital survival.

1. Introduction

Hospital-acquired infections (HAIs) represent one of the most significant threats for patients admitted to hospital [1]. A recent multistate point-prevalence survey involving 183 hospitals in the US reported that 4% of hospitalized patients developed an HAI, with *Clostridium difficile* and ESKAPE pathogens (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa*, and *Enterobacteriaceae*) being most frequently isolated [2]. Hospitals and other healthcare settings may serve as reservoirs for antimicrobial-resistant (AMR) ESKAPE pathogens, allowing these opportunistic pathogens to spread from the hospital environment to inpatients, or occasionally to the community via the movement of individuals in and out of the hospital. In fact, recent epidemiological studies confirmed the prevalence of *S. aureus*, *Acinetobacter baumannii* and *P. aeruginosa* on various hospital surfaces, including bed rails, armrests and nurse call buttons, all found within intensive care units and frequently touched by medical practitioners [3,4].

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Breaking the transmission of ESKAPE pathogens in hospital settings using multifaceted infection prevention and control programs is considered an effective strategy to prevent HAIs [5,6]. Environmental cleaning programs combining disinfectants, detergents and ultraviolet (UV) radiation are applied in most hospital settings and appear to be insufficient in eradicating microbial contamination. Despite the compliance of current surface disinfection and cleaning programs, ESKAPE pathogens continue to be isolated from the hospital environment [2]. For example, a recent study found that 27% of hospital rooms remained contaminated with *A. baumannii* or methicillin-resistant *S. aureus* (MRSA) following 4 rounds of bleach disinfection [7]. The failure of such methods in eliminating surface contamination might be due to the high resistance of bacterial cells to these adverse conditions as an evolutional consequence, and/or by adopting unique survival strategies such as biofilm formation and encapsulation.

The presence of dry microbial biofilms has recently been demonstrated on many hospital surfaces in diverse settings [8]. Biofilms, consisting of microorganisms embedded in exopolymeric substances (EPS), are extremely difficult to eliminate due to their increased resistance to detergents and disinfectants. Such microbial communities can periodically release free-living planktonic bacteria into the environment that may act as an infectious nidus [9,10]. It has now been confirmed that microbial biofilms on hospital surfaces contribute to the transmission of hospital pathogens to inpatients and play an important role in the occurrence of HAI [11–13]. In addition to dry biofilms, capsule production is considered a microbial strategy that also allows prolonged survival of Gram-negative pathogens such as *Klebsiella* spp. and *Acinetobacter* spp. in the hospital environment [14]. Bacterial capsule and biofilms often share compositional and structural similarities, with polysaccharide and water as their major components. It remains unclear which strategy plays a more important role in bacterial survival in the hospital environment.

This study aimed to compare the importance of biofilm formation and capsule production in shielding bacterial pathogens from adverse hospital conditions such as the presence of disinfectants, desiccation, and UV radiation. Furthermore, the correlation between these two important strategies underpinning bacterial survival on hospital surfaces was investigated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Capsule-positive, biofilm-positive *Acinetobacter baumannii* AB5075 (wild type, WT) and *Klebsiella pneumoniae* ATCC 43816 (WT), and their isogenic, capsule-deficient transposon insertion mutants were selected for this study (Table 1). These strains were selected as well-characterised, virulent exemplars of these pathogenic species, for which complete capsule structures and genome sequences were available. Transposon-insertion mutants were obtained from the *A. baumannii* AB5075 three-allele library, and from our previous work, respectively [15–17]. Other well-known biofilm-producing bacterial strains, including *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* RP62A, and *Pseudomonas aeruginosa* PAO1 were also included to verify the importance of biofilm formation in bacterial survival in the hospital environments (Table 1). All bacterial strains were stored in glycerol at −80 °C and grown on Nutrient agar at 37 °C as required.

2.2. Planktonic and colony biofilm growth models using mixed cellulose ester (MCE) membranes

Mixed cellulose ester membranes (MCE, 0.22 μm, MF-Millipore) were selected as the substratum to support planktonic and biofilm growths of bacterial cells. This was to mimic the solid-air interface at which bacterial cells interact with dry hospital surfaces. Bacterial colonies were resuspended to ~1 × 10^7 CFU/mL in Mueller-Hinton (MH) broth and 20 μL was added onto an MCE membrane and incubated on MH agar at 37 °C overnight. For comparison, a planktonic culture was also established using the MCE membrane; the membrane with bacterial suspension was directly placed in a Petri dish without agar or further growth.

Table 1
Bacterial strains used in this study.

Species	Strain	Characteristics/phenotype
Acinetobacter baumannii	AB5075 [63]	WT mouse-virulent, capsule-positive strain
	AB5075 tn::*wzc* [17]	Capsule-deficient transposon insertion mutant
	AB5075 *gacA*::tn26	Capsule-reduced transposon insertion regulatory mutant
Klebsiella pneumoniae	ATCC 43816 [64]	WT mouse-virulent, capsule-positive strain, Sequence type ST66, capsule type K2
	ATCC 43816 ins-*wcaJ* [15]	Capsule-deficient transposon insertion mutant
	ATCC 43816 ins-*wza* [16]	Capsule-deficient transposon insertion mutant
Pseudomonas aeruginosa	PAO1	Laboratory reference strain, biofilm-positive, capsule-negative
Staphylococcus aureus	ATCC 25923	Laboratory reference strain, biofilm-positive, capsule-negative
Staphylococcus epidermidis	RP62A	Laboratory reference strain, biofilm-positive, capsule-negative

2.3. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to confirm the free-living status of planktonic cells and the presence of densely grown cells and extracellular polymeric substances (EPS) of biofilms. Bacterial cells on the MCE membranes were fixed with 100 μL of 2.5% glutaraldehyde at 4 °C overnight. The MCE membranes were then placed on a sterile tissue paper to remove excess solution present in the membrane. Bacterial cells on the MCE membranes were dehydrated with 100 μL of 50%, 75%, 90% and 100% (absolute) ethanol for 10 min respectively; a tissue paper was used to absorb excess ethanol before adding the subsequent drop of ethanol of higher concentration. The MCE membranes were left to air dry overnight, followed by gold coating using the Bal-Tec SCD-005 sample sputter coater. Samples were imaged using the FeiNova NanoSEM FEGSEM.

2.4. Validation of capsule production

It should be noted that *A. baumannii* tn: wza mutant strain was presumed to fail to transport synthesized capsular polysaccharide from the periplasm space to the surface of the bacterium and the tn:*gacA* was a regulator mutant that was only predicted to produce less capsule [18,19]. The capsule-producing phenotype of *A. baumannii* and *K. pneumoniae* WT and mutant strains was thus validated using the gradients method as previously described [20,21]. In brief, an overnight culture of bacteria was overlaid onto Percoll gradients (800 μL of bacterial culture, 60% and 40% Percoll for *A. baumannii* WT and tn:*gacA* comparison; 600 μL of bacterial culture, 100% and 60% for all others). The tubes were centrifuged at 5500 rpm for 5 min to allow migration of bacterial cells. All Percoll gradient experiments were performed in three biological repeats.

2.5. Desiccation assay

To evaluate the effect of capsule production and biofilm formation on bacterial tolerance to desiccation, MCE membranes with planktonic cells or colony biofilms were placed in a desiccator for 0, 1, 3, 7, 28 days respectively. At each time point, planktonic/biofilm-containing MCE

membranes were removed from the desiccator and placed into a 15 mL FALCON tube containing 5 mL phosphate-buffered saline (PBS), followed by vortex using a multi-vortex mixer at maximum speed for 2 min and sonication for 10 min in a sonication bath (frequency = 42 kHz). The number of survivor cells was determined by colony-forming unit (CFU) enumeration. This assay was performed in three biological repeats in technical duplicate.

2.6. Benzalkonium chloride resistance assays

Benzalkonium chloride (BAC) was chosen as a representative of medical-grade disinfectants. Minimum inhibitory concentrations (MICs) of benzalkonium chloride were determined using the standard broth microdilution method recommended by the Clinical and Laboratory Standard Institute (CLSI) [22]. This assay measured the BAC susceptibility of bacteria grown as planktonic cells and was performed in three biological repeats in technical duplicate. Biofilm resistance to BAC was measured using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay [23]. To mimic the liquid-solid-air interface that bacteria may encounter when a disinfectant solution is used on hospital surfaces, biofilms were pre-formed in a 96-well microplate and washed with PBS as previously described [23]. One hundred and 20 μL of BAC solutions at concentrations ranging from 16 μg/mL to 256 μg/mL were added to biofilm-containing microwells. The microplate was incubated statically at 37 °C for 25 min to provide sufficient time for BAC-mediated killing, followed by washing with 120 μL PBS to remove residual BAC. The PBS in the microwells was then replaced with 120 μL XTT-menadione solution consisting of 0.5 mg/mL XTT. The 96-well microplate was further incubated at 37 °C for 1 h. After incubation, 100 μL of solution was transferred to a new 96-well microplate, and the absorbance of each microwell was measured using a Tecan Infinite M200 plate reader at 492 nm. The lowest BAC concentration that yielded at least 75% growth inhibition denoted the BAC biofilm MIC75 ($BMIC_{75}$) of a given bacterial strain. This assay was performed in three biological repeats with technical duplicates.

2.7. Resistance to ultraviolet (UV) radiation

To determine bacterial resistance to ultraviolet (UV) radiation, planktonic cultures and biofilms grown on MCE membrane were exposed to an ultraviolet light in a class II biological safety cabinet for 30s, 1 min and 15 min respectively. The number of surviving cells was determined by CFU enumeration. This assay was performed in three biological repeats with technical duplicates.

2.8. Interaction between biofilm formation and capsule production

Interaction between biofilm formation and capsule production was investigated by quantitatively and qualitatively comparing biofilms formed by *K. pneumoniae* and *A. baumannii* WT and capsule-deficient mutant strains. Biofilms grown in 96-well microplates were examined for their biomass using crystal violet staining, as previously described [24]. This assay was performed in four biological repeats with technical triplicates. For qualitative assessment, biofilms were grown on medical grade silicone disks in a 24-well microplate for 24 h and examined using SEM, as previously described [23].

2.9. Cell surface hydrophobicity assessment

The cell surface hydrophobicity (CSH) of the WT and capsule-deficient mutant strains of *K. pneumoniae* and *A. baumannii* were assessed using the p-xylene assay, as described by Krasowska and Sigler (2014) [25]. Percentage relative hydrophobicity was calculated. This assay was performed in three biological repeats with technical duplicates.

2.10. Statistical tests

All raw data were checked for normality using the Shapiro-Wilk test. Normally distributed data were presented as mean ± standard error of the mean (SE). Statistical analysis of the desiccation assays, which had two independent variables (strain and time), were carried out using an ordinary two-way ANOVA. Differences between UV results were analyzed using the Šídák's multiple comparisons test. An unpaired *t*-test with Welch's correction was used for data for biofilm quantitative assays and the cell surface hydrophobicity assays. *p*-values <0.05 were considered significant. All statistical tests were performed in GraphPad Prism 9 (GraphPad Software LLC).

3. Results

3.1. Validation of planktonic and biofilm growth modes of bacteria on MCE membranes

Colony biofilms grown on MCE membranes were used to represent biofilms on dry hospital surfaces as both are initiated by bacterium-surface interactions at a solid-air interface. Planktonic cultures were also grown on MCE membranes for comparison, and to avoid rapid formation of adherent monolayers when cultured in a tissue culture treated polystyrene (TCPS) microplate [24]. The maintenance of bacteria in planktonic and biofilm states on MCE membranes were validated using high-resolution SEM, with Gram-positive *S. aureus* ATCC 25923 and Gram-negative *A. baumannii* AB5075 as the representative strains. Planktonic cells grown on the MCE membrane presented as sporadic single cells and occasionally small clusters (Fig. 1A). The non-adherent status of planktonic cells on the MCE membranes was supported by our observation that a single *S. aureus* cell changed its position on the MCE membrane when the chamber pressure of the FeiNova NanoSEM was changed from $\sim 1.2 \times 10^{-5}$ mbar to $\sim 7.5 \times 10^{-6}$ mbar during re-focusing and re-imaging (Fig. 1B). In contrast, colony biofilms grown on MCE membrane had an enormous amount of densely-grown bacterial cells interconnected with EPS, showing a typical *in vitro* biofilm structure (Fig. 1C).

3.2. Phenotypic validation of capsule-deficient mutant strains

Before assessing each strain for hospital survival, capsule production of *A. baumannii* transposon insertion mutants (tn:*wzc* and tn:*gacA*) and *K. pneumoniae* mutants (ins-*wcaJ* and ins-*wza*) were phenotypically examined. Using Percoll gradients, the *A. baumannii* tn:*wzc* and tn:*gacA* cells (Fig. 2A) and *K. pneumoniae* ins-*wcaJ* and ins-*wza* mutants (Fig. 2B) readily disseminated throughout the 40% (for tn:*gacA* cells) or the 60% (for all other mutants) Percoll layer, indicating a capsule-deficient nature, whilst the encapsulated WT cells remained at the top fraction.

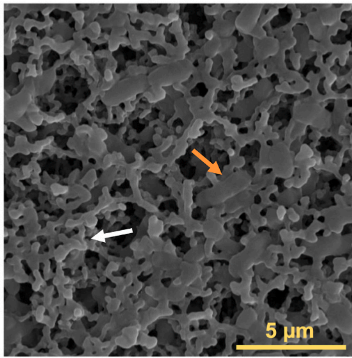
3.3. Biofilm formation plays a more important role than capsule production in bacterial tolerance to environmental desiccation

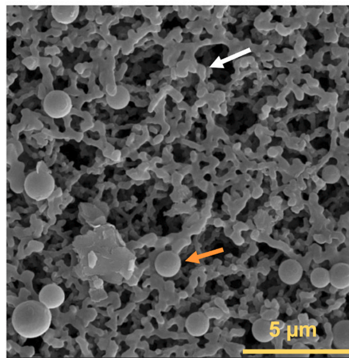
When grown as planktonic cells, both *A. baumannii* and *K. pneumoniae* WT strains demonstrated drastic decreases in CFU enumeration under environmental desiccation, with an ~2-log reduction and ~4-log reduction respectively after 7-day treatment (Fig. 3A and B). Qualitative SEM of post-desiccation planktonic growths (Fig. 3E) supported our CFU enumeration results, with most *K. pneumoniae* cells on the MCE membranes showing shriveled appearance and *A. baumannii* presenting cell debris. In contrast, biofilm growth of *A. baumannii* and *K. pneumoniae* conferred significant protection to bacterial cells, with no reduction of CFU enumeration even after 28-day desiccation (Fig. 3C, D and 3E).

Comparing the desiccation-survival profiles between the WT and capsule-deficient mutants under planktonic condition, both tn:*wzc* and tn:*gacA* strains of *A. baumannii* showed higher sensitivity to desiccation

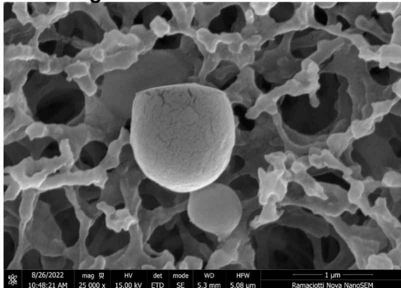
A) Planktonic culture

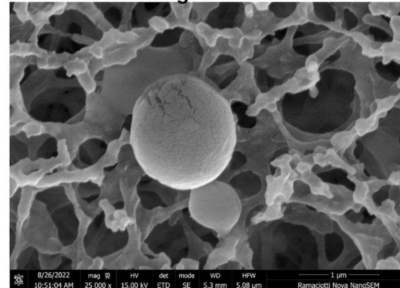
A. baumannii (WT) *S. aureus*





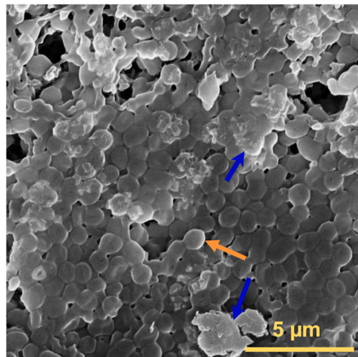
B) First image at 10:48 am Refocused/reimaged at 10:51 am





C) Biofilms

A. baumannii (WT) *S. aureus*



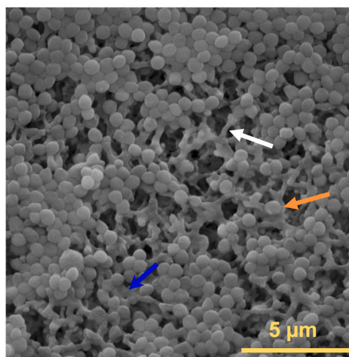


Fig. 1. SEM validation of planktonic cells and biofilms grown on MCE membranes. A) Planktonic mode characterized by individual cells (red arrows) on the mesh-like structure of the MCE membrane (white arrows). B) Free living status of planktonic cells on the MCE membrane was further verified using SEM at a very high magnification and by changing the chamber pressure. C) The biofilm growth mode was verified by densely grown bacterial cells embedded in EPS matrix (blue arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

relative to their WT strain ($p < 0.0001$) throughout the entire 28-day desiccation period (Fig. 3A), with no viable cells detected on Day 28. Despite a >3 log reduction in CFU enumeration, WT *A. baumannii* cells were still detectable after the28-day desiccation. On the contrary, the *K. pneumoniae* strains ins-*wcaJ* ($p = 0.0218$) and ins-*wza* ($p = 0.0046$) demonstrated increased survival relative to the WT strain, suggesting higher resistance to desiccation of these capsule-deficient mutants (Fig. 3B). Although minor but significant differences were observed between WT and capsule-deficient mutants of *A. baumannii* and *K. pneumoniae* when grown as biofilms, all strains showed high resistance to environmental desiccation, with less than 1.2 log reduction in CFU enumeration after 28-day desiccation (Fig. 3A and B).

3.4. Biofilm formation, but not capsule production, increases bacterial resistance to BAC disinfection

Using CLSI-recommended broth microdilution assay for planktonic cells, *A. baumannii* and *K. pneumoniae* had MICs to BAC of 32 and 64 μg/mL respectively, with no difference between WT and capsule-deficient mutant strains (Table 2). XTT assay revealed that biofilms formed by WT *A. baumannii* and *K. pneumoniae* had BAC $BMIC_{75}$ of 64 μg/mL compared to 128 μg/mL for their isogenic capsule-deficient mutants (Table 2), only marginally higher than the CLSI MICs for their planktonic counterparts (≤2-fold). It was speculated that biofilm resistance to BAC might have been complicated by the intrinsic BAC resistance of *A. baumannii* and *K. pneumoniae* strains used in this study. We thus introduced other biofilm-forming bacterial species that were sensitive to BAC into this study to clarify the role of biofilm formation on BAC resistance. Biofilms formed by *S. aureus* ATCC 25923 and *S. epidermidis* had higher resistance to BAC than their planktonic counterparts, supported by high biofilm MIC_{75} of 64 μg/mL and 128 μg/mL respectively, relative to CLSI MIC of 4 μg/mL for planktonic cultures. Another Gram-negative bacterium *P. aeruginosa* PAO1 also showed at least a 4-fold increase in MICs when its growth mode changed from planktonic cells to biofilms (Table 2).

3.5. Biofilm formation plays a more important role in bacterial survival against UV exposure

UV radiation has been widely implemented as an important

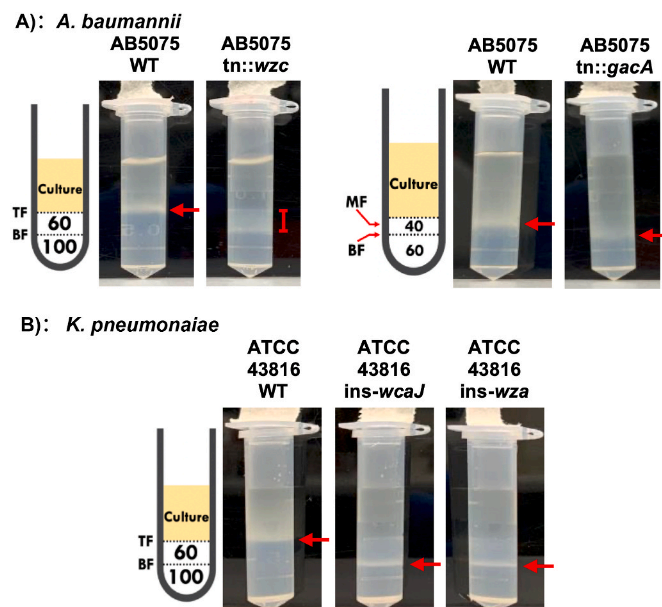


Fig. 2. Phenotypic validation of capsule production of *A. baumannii* and *K. pneumoniae* WT and mutant strains. Overnight cultures were placed in a Percoll gradient containing 60% and 100% or 60% and 40% Percoll layers. Loss of capsule in the *A. baumannii* (A) and *K. pneumoniae* (B) mutants was confirmed by increased bacterial migration through Percoll gradients (after centrifugation) when compared to their WT parent strain. Cells are visualised as an opaque white band indicated by the red arrows/bars. TF = top fraction, MF = middle fraction, BF = bottom fraction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

disinfection procedure in the hospital environment. In comparison of UV survival profiles between WT and capsule-deficient mutants when grown as planktonic cultures, *A. baumannii* WT presented a lower viability than its *tn::wzc* or *tn::gacA* mutants upon receiving 1 min of UV irradiation ($p = 0.0021$ and $p < 0.001$ respectively, Fig. 4A). Similarly, *K. pneumoniae* WT had lower viability following on minute UV irradiation than either of its capsule-deficient mutant strains ins-*wcaJ* ($p = 0.0419$) and ins-*wza* ($p < 0.001$) (Fig. 4B). Nevertheless, 15 min of UV radiation eradicated all WT and mutants of *A. baumannii* and *K. pneumoniae* grown as planktonic cells. In contrast, all *A. baumannii* and *K. pneumoniae* biofilms remained intact throughout the entire 15-min of UV exposure, regardless of strain (Fig. 4Cs and 4D).

3.6. Capsule production negatively impacts biofilm formation

Semi-quantitative microplate-based biofilm assay in combination with crystal violet staining demonstrated a significantly lower OD_{600} of WT *K. pneumoniae* than that of capsule-deficient mutant strains ins-*wcaJ* ($p = 0.024$) and ins-wza ($p = 0.019$) (Fig. 5A). The opposite trend was observed for *A. baumannii,* with the OD_{600} for its WT biofilms being significantly higher than that of the tn:*wzc* ($p < 0.001$) and tn:*gacA* ($p = 0.03$) mutant strains. As crystal violet also non-specifically binds to negatively-charged capsule components of *A. baumannii*, OD readings for the WT biofilm might have been artificially amplified. To gain a direct insight of the relationship between capsule production and biofilm formation, we further employed the qualitative SEM to examine biofilm structure grown on a medical-grade silicone disk. High-resolution SEM showed that all *A. baumannii* and *K. pneumoniae* capsule mutants produced robust biofilms containing densely packed bacterial cells on silicone disks (Fig. 5B), whilst their isogenic WT strains only grew as monolayer biofilms or small clusters (Fig. 5B).

CSH is a crucial factor facilitating the initial adhesion of bacterial cells to a hydrophobic surface and was believed to be an important mediator for capsule production and biofilm formation [25]. We tested the affinity of the WT and capsule mutant strains to p-xylene for their CSH. *A. baumannii* mutant strains tn:*wzc* and tn:*gacA* had significantly higher surface hydrophobicity (88% and 62.9% affinity to p-xylene) than the WT (35.7% affinity; Fig. 5C). *K. pneumoniae*, however, showed no difference in cell surface hydrophobicity between its capsule-deficient mutants ins-*wcaJ* (27.6% affinity; $p = 0.3771$) and ins-*wza* (30.6% affinity; $p = 0.0758$) and their WT strain (25.6% affinity; Fig. 5D).

3.7. The vital role of biofilm formation on bacterial survival can be extended to other bacterial species frequently encountered in the hospital environment

Biofilms formed by *S. aureus* ATCC 25923*, S. epidermidis* RP62A, and *P. aeruginosa* PAO1 were compared with their planktonic counterparts for the role of biofilm formation in hospital survival. Similar to that for *A. baumannii* and *K. pneumoniae,* changing growth modes from planktonic cultures to biofilms significantly increased bacterial resistance to long-term environmental desiccation and UV radiation (Figs. S1–2).

4. Discussion

Hospital-acquired infections, particularly those caused by AMR pathogens residing in the hospital environment, remain a critical medical issue due to their high mortality rates. Breaking the transmission of AMR pathogens in hospital settings has been recognized as an effective strategy to prevent AMR and HAIs [5]. Capsule production and biofilm formation are the two mechanisms contributing to bacterial survival in the hospital environment. The role of biofilms in bacterial survival on dry hospital surfaces has been well studied [26–28], though the impact of capsule production and its relationship with biofilm formation in bacterial hospital survival is yet to be understood. This study aimed to determine how the two microbial strategies, collaboratively or counteractively, contribute to bacterial survival on dry hospital surfaces under various adverse conditions. Our key findings include 1) Biofilm formation played a vital role in bacterial survival against environmental desiccation, disinfectants and UV radiation, 2) Encapsulation provided a relatively minor protection against desiccation, no protection against disinfectants and might sensitize bacterial cells to UV radiation, and 3) Encapsulation of bacterial cells compromised their biofilm formation, possibly by reducing the cell surface hydrophobicity or via other mechanisms.

Biofilm formation is considered the "default growth mode" across many bacterial species in the hospital environment [29,30]. Microbial biofilms were often studied in parallel with their planktonic counterparts for the purpose of comparison. A major technical challenge for this study was to grow planktonic cultures for the desiccation and UV radiation assays. While other studies have used TCPS 96-well microplates to investigate planktonic cell resistance to desiccation [31,32], the hydrophobic TCPS surfaces support a rapid conversion of free-living cells to adherent monolayers [24]. Adherent monolayers are an intermediate growth mode between planktonic cells and biofilms and may present characteristics similar to biofilms [24]. MCE membranes have porous and hydrophilic properties and are able to minimize non-specific binding of bacterial cells to the surface [33,34]; the planktonic cultures grown on MCE membranes in this study showed free-living single cells, capturing the definition of a "planktonic state". This model, along with the MCE membrane-based colony biofilm model, also closely mimicked the solid-air interface encountered on the dry hospital surfaces. Other researchers have used the CDC biofilm reactor to generate "hydrated" biofilms that were subsequently dehydrated to represent biofilms found in the hospital environment [35]. Chng et al. (2020) recently found higher abundancies of common nosocomial pathogens in numerous "dry" hospital sites, such as bed rail, bedside locker, cardiac table, door handle, and pulse oximeter, relative to that of the sink trap or aerator

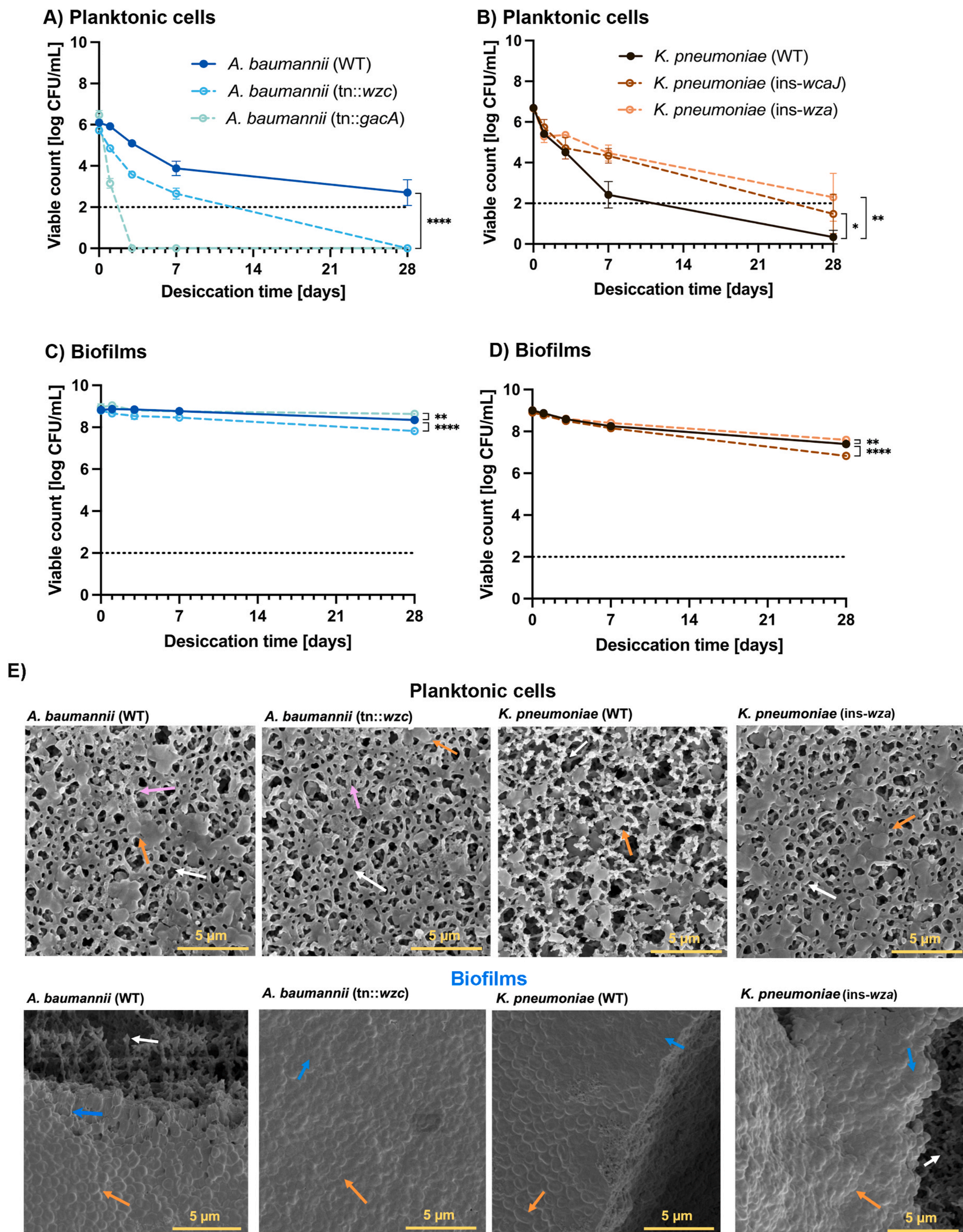


Fig. 3. Desiccation survival profiles of WT and capsule-deficient mutant strains of *A. baumannii* and *K. pneumoniae*. Planktonic cell (A & B) and biofilms (B & D) of WT and capsule-deficient mutant strains of *A. baumannii* and *K. pneumoniae* were placed in desiccators and sampled on days 0, 1, 3, 7 and 28 for viable bacteria. Each data point represents the average survival across three biological replicates with technical duplicates, with its corresponding ± SE shown in an error bar. The black horizontal dotted line denotes the 2-log CFU/mL detection limit. An ordinary 2-way ANOVA was used to assess the statistical significance between the overall desiccation profiles between WT and capsule mutant *A. baumannii* and *K. pneumoniae*. **** = $p < 0.0001$, ** = $p < 0.01$, * = $p < 0.05$. E. SEM of planktonic and biofilm cells of WT and capsule-deficient mutant strains of *A. baumannii* and *K. pneumoniae* after desiccation for 7 days. White arrows, MCE membrane; orange arrows, individual bacterial cells; purple arrows, cell debris; blue arrows, biofilm EPS matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Benzalkonium chloride susceptibility of nine different bacterial strains grown as planktonic cells or biofilms.

Microorganism	Broth microdilution Planktonic MIC (μg/mL)	XTT
		Biofilm MIC_{75} (μg/mL)
A. baumannii (WT)	32	64
A. baumannii (tn::*wzc*)	32	128
A. baumannii (tn::*gacA*)	32	128
K. pneumoniae (WT)	64	64
K. pneumoniae (ins-*wcaJ*)	64	128
K. pneumoniae (ins-*wza*)	64	128
P. aeruginosa (PAO1)	128	>256
S. aureus (ATCC 25923)	4	128
S. epidermidis (RP62A)	4	64

[3]. The CDC biofilm reactor model may closely mimic biofilms grown in a sink trap or aerator in hospital wards; bacterial cells grown on "dry" hospital sites are less likely to receive such a strong liquid shear force, a unique condition provided by the CDC biofilm reactor. Membrane-based colony "dry" biofilms and microplate-based "wet" biofilms were thus set up to represent those found in "dry" hospital environments.

Desiccation generates an imbalance between cell volume and the plasma membrane surface area, inducing protein aggregation and the production of reactive oxygen species (ROS) that further deactivate bacterial cells [36]. Bacteria may utilize multiple strategies to combat desiccation, including capsule production and biofilm formation. The efficacy of the former strategy was demonstrated in *A. baumannii* in this study, given that the loss of functional *wzc* resulted in attenuated bacterial resistance to desiccation. This observation was consistent with findings of several other studies that also used capsule-deficient *A. baumannii* [31,37]. The extent of capsule-mediated protection, appeared to be species-specific, as *K. pneumoniae* mutant strains ins-*wcaJ* and ins-*wza* exhibited higher resistance to desiccation than their WT. The higher bacterial survival rate of capsule-deficient *K. pneumoniae* when facing desiccation might be due to secondary defects aside from capsule loss [38]. It was possible that both ins-*wcaJ* and ins-*wza* mutants had decreased metabolism as a secondary defect, as supported by lower levels of cell fitness (Fig. S3); a relatively "dormant" cell state has been reported to promote bacterial tolerance to desiccation [39].

Our study confirmed the role of biofilm formation in promoting bacterial resistance to desiccation, a notion observed in other studies [40]. Our work also deduced that biofilm formation conferred more protection than capsule production, based on our finding that biofilms formed by capsule-deficient strains gained the same level of resistance to desiccation when grown as biofilms as their WT counterparts. Structural comparisons between bacterial capsules and biofilms might explain the difference. Both bacterial capsule and biofilm EPS matrix contain water as their major component protecting cells from desiccation. The width of bacterial capsules ranges between 2 nm and 10 μm [41], while the EPS matrix of dry biofilms could reach a thickness of 24 μm–47 μm [35]. A thicker and sturdier "biofilm wall" allows bacterial cells to retain intracellular water more effectively and thus, maintain their high resistance to environmental desiccation.

In agreement with other published work, our study found that biofilm formation rendered bacterial cells highly resistant to disinfectants. Capsule production however, had little effect on disinfectant resistance, as identical BAC MICs were found for the WT and capsule mutant strains. This is contrast to that reported by Tipton et al. (2018) who previously reported that the loss of functional *wzc* resulted in decreased resistance to BAC in *A. baumannii* AB5075 [31]. It should be noted though, that Tipton et al. (2018) used a CFU enumeration method that was able to detect a marginal difference in antimicrobial resistance rather than a broth microdilution as used in the current study. Another limitation of this study is that CLSI MICs and $BMIC_{75}$ have different endpoints that may compromise the solidity of our conclusion. Nevertheless, it is clear that capsule production does not substantially increase resistance of bacteria against BAC while biofilm formation does equip bacterial cells with high resistance.

Biofilm formation completely mitigated the impact of UV disinfection, as supported by negligible loss of cell viability following UV exposure. This agrees with results from another published study that was conducted for water distribution systems [42]. It is possible that the anti-UV effects of biofilms are likely lent by the thick biofilm EPS matrix, which limits UV penetration towards the embedded bacterial cells.

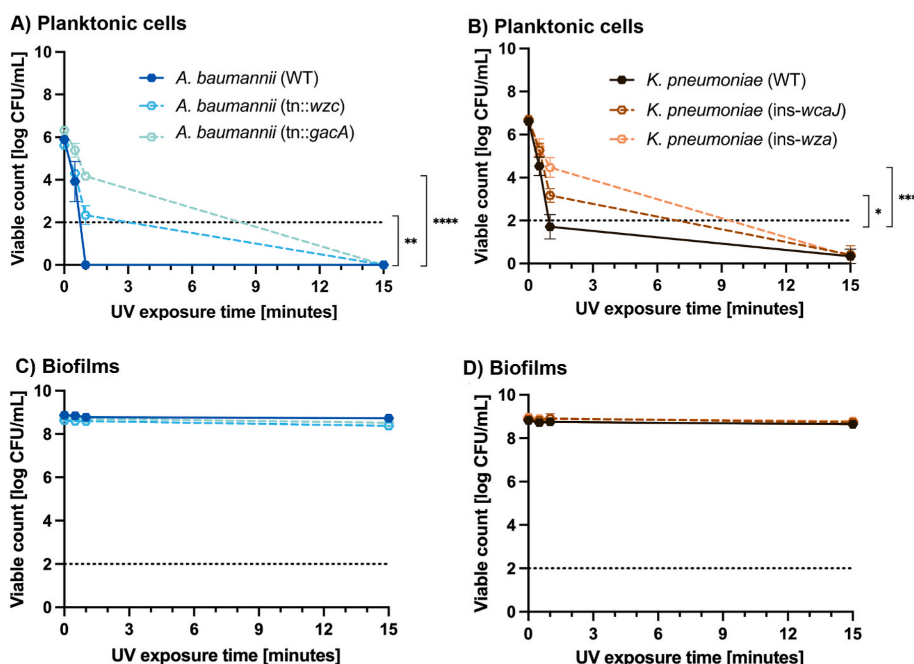


Fig. 4. Survival profiles of *A. baumannii* and *K. pneumoniae* WT and capsule-deficient mutant strains against UV radiation. Planktonic cells (A & B) and biofilms (C & D) of *A. baumannii* WT, tn:*wzc* and tn:*gacA* mutant strains and *K. pneumoniae* WT, ins-*wcaJ* and ins-*wza* mutant strains were exposed to UV radiation with survival assessed at 0, 0.5, 1 and 15 min. Each data point represents the average survival across three biological replicates with technical duplicates, with its corresponding ± SE shown in an error bar. The black horizontal dotted line denotes the 2-log CFU/mL detection limit. A Šídák's multiple comparisons test was used to assess the statistical significance between WT and capsule-deficient mutant strains at the 1-min timepoint. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

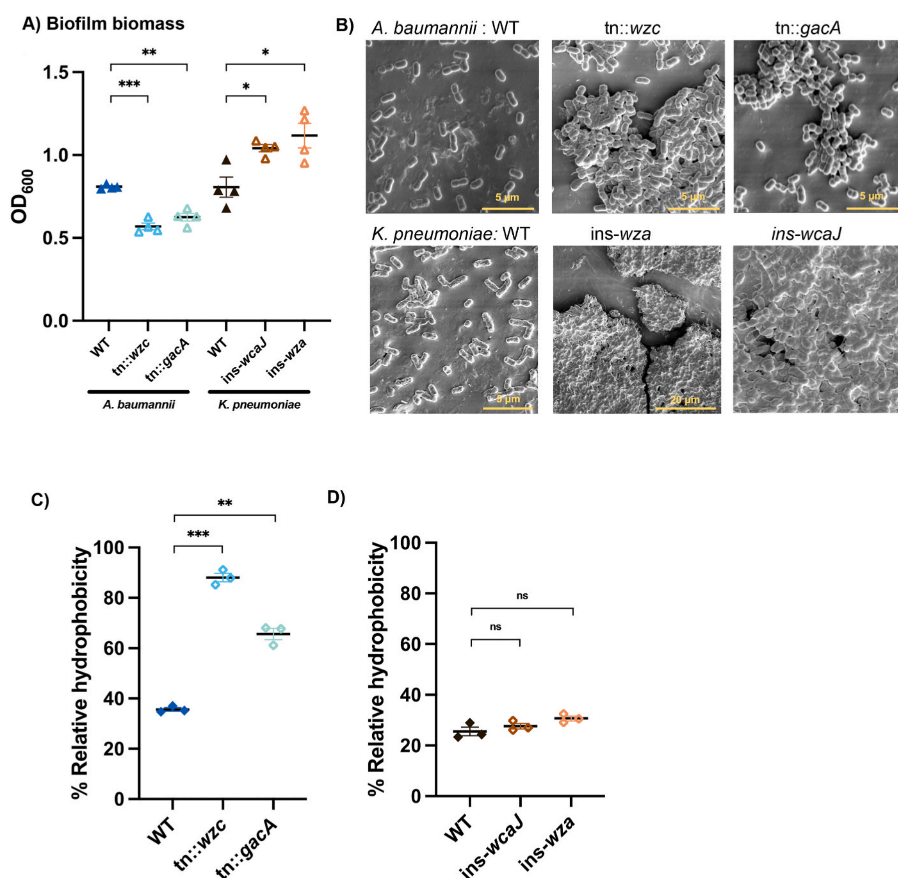


Fig. 5. Biofilm formation and surface hydrophobicity of *A. baumannii* and *K. pneumoniae* WT and capsule-deficient mutant strains. A) Biomass of biofilms formed by *A. baumannii* and *K. pneumoniae* WT and capsule-deficient mutant strains in 96-well microtiter plates were quantified using 0.1% crystal violet staining. All data points represent a single biological replicate averaged from three technical replicates. The mean and ±SE are shown in a black horizontal line and an error bar, respectively. An unpaired *t*-test with Welch's correction was used to assess statistical significance. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. B) Qualitative and structural assessment of biofilm formation by *A. baumannii* and *K. pneumoniae* WT and capsule-deficient mutant strains on medical-grade silicone disks, using SEM. Structures of WT and capsule mutant *A. baumannii* and *K. pneumoniae* biofilms were qualitatively assessed using SEM. C) Cell surface hydrophobicity analysis of *A. baumannii* WT and capsule-deficient mutant strains, D) Cell surface hydrophobicity analysis of *K. pneumoniae* WT and capsule-deficient mutant strains. All data points represent a single biological replicate averaged from technical duplicates, with the mean and ±SE shown as a black horizontal line and error bars, respectively. An unpaired *t*-test with Welch's correction was used to assess statistical significance. *** = $p < 0.001$, ** = $p < 0.01$, ^{ns} = $p > 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Surprisingly, capsule production promoted the effectiveness of UV radiation against *A. baumannii* and *K. pneumoniae*, rather than protecting bacterial cells from UV-mediated damage. To the best of our knowledge, this is the first study examining the linkage between capsule production and bacterial resistance to UV radiation. The exact mechanism by which capsule promotes susceptibility to UV is currently unknown. It is speculated that the capsule absorbs more UV radicals that contributes to more UV damage of its target, the bacterial DNA [43]. The ESKAPE pathogens isolated from the hospital environment often exhibit an encapsulated phenotype and higher virulence [44,45]. Given the abundance of encapsulated bacteria in the hospital environment, the value of UV radiation in hospital disinfection may be under-estimated.

The interaction between biofilm formation and capsule production may add complexity to the overall bacterial survival in the hospital environment. We and others have found that capsule production had an adverse impact on biofilm formation [46,47]. In a bacterial cell, losing the hydrophilic capsule may expose the hydrophobic cell membrane, as a result, non-encapsulated cells can readily adhere to abiotic surfaces due to matching hydrophobic properties [25]. Therefore, the increased biofilm formation of a capsule-deficient mutant is at least partially attributed to changes in bacterial CSH [48], which was observed in *A. baumannii* in the current study. This notion, however, appeared to species-specific, given that the CSH between WT and capsule mutant *K. pneumoniae* were similar, as found by us and others [49,50]. We speculated that loss of capsule in *K. pneumoniae* might expose the type 3 fimbriae, an adhesin that is buried in the capsule [51]. The presence of fimbriae allows bacteria to attach optimally to an abiotic surface and facilitate biofilm formation [51]. The role of capsule production in biofilm biology might be more complex than the simplistic view we presented [52]. Enhanced capsule synthesis has been reported at the late stage of biofilm formation in several bacterial species, implicating an incompletely defined role of capsule production in the full life cycle of biofilm formation [52]. At the maturation phase, capsule production appeared to be important for the maintenance of biofilm size and dispersal, with quorum sensing as the key driver [53]. It is also possible that mixed biofilm growth of encapsulated and non-encapsulated bacterial strains confers protection to the more "vulnerable" capsulated strains against hospital environmental stressors such as UV radiation; future research is warranted. It should also be noted that growth of *A. baumannii* and *K. pneumoniae* capsule-positive WT on silicone disks (Fig. 5B) should still be categorized as monolayer biofilms; future assessment of the resistance of such biofilms against UV radiation, disinfectants and environmental desiccation is needed.

A limitation of our study was that we only investigated the role of capsule production of Gram-negative bacteria in hospital survival. Many Gram-positive bacterial and fungal pathogens such as *S. aureus*, *Streptococcus pneumoniae* and *Cryptococcus neoformans* also produce capsular polysaccharides [54–56]. In line with what was observed for *A. baumannii* and *K. pneumoniae*, capsule-deficient *S. aureus* and *S. pneumoniae* were reported to form biotic monolayers or abiotic biofilms to a greater extent than their capsule-positive counterparts [57, 58]. In Gram-positive bacteria, capsule production is an important biological process for cell wall integrity, coordinating with the biosynthesis of other key components such as peptidoglycan and wall teichoic acid [59]. Formation of these cell wall components utilizes a pool of essential precursors including UDP-N-acetyl-glucosamine, which is also the intermediate precursor to polysaccharide intercellular adhesin (PIA) of S. epidermidis biofilms and polymeric β-1,6-linked N-acetylglucosamine (PNAG) of S. aureus biofilms [60,61]. It is possible that formation of staphylococcal polysaccharide capsule and biofilm EPS compete for the supply of the same sugar nucleotide [61]. Other bacterial species can also use common precursors for synthesis of both capsules and other biofilm matrix polysaccharides; these pathways are reviewed in detail in Whitfield et al. (2020) [62].

In summary, our study established that biofilm formation, but not capsule production, contributes significantly to hospital survival of bacterial cells. Capsule production may promote bacterial sensitivities to UV radiation and reduces bacterial capability of forming biofilms on abiotic surfaces, adversely affecting bacterial survival in the hospital environment. Due to the prevalence of encapsulated ESKAPE pathogens in hospitals, further investigation of hospital dry biofilm prevention strategies is strongly encouraged as it may eradicate the source of pathogens causing HAIs.

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CRediT authorship contribution statement

Charles Nunez: Experimentation, Methodology, Validation, Writing – original draft. **Xenia Kostoulias:** Supervision, Experimentation, Methodology, Validation, Writing – review & editing. **Anton Peleg:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Francesca Short:** Conceptualization, Funding acquisition, Supervision, Experimentation, Methodology, Validation, Writing – review & editing. **Yue Qu:** Conceptualization, Supervision, Experimentation, Methodology, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francesca Short reports financial support was provided by the Australian Research Council.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2023.100105.

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