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# Biofilm formation in *Acinetobacter baumannii*

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**SUMMARY**

*Acinetobacter baumannii* has received much attention in recent years because of its increasing involvement in a number of severe infections and outbreaks occurring in clinical settings, and presumably related to its ability to survive and persist in hospital environments. The treatment of infections caused by *A. baumannii* nosocomial strains has become increasingly problematic, due to their intrinsic and/or acquired resistance to multiple classes of antibiotics. Furthermore, the demonstrated ability of nosocomial strains to grow as biofilm is believed to play a significant role in their persistence and antibiotic resistance.
This review summarises current knowledge on *A. baumannii* biofilm formation and its clinical significance, as well as the related genetic determinants and the regulation of this process.

*KEY WORDS:* Biofilm, *Acinetobacter baumannii*, Biofilm-related genes.

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## INTRODUCTION

Initially considered of scarce clinical interest, *A. baumannii* is now among the most important nosocomial pathogens, because of its increasing isolation in clinical settings, where it causes a wide range of infections (bloodstream and urinary tract infections, ventilator-associated pneumonia, wound, skin and soft-tissue infections) often associated with high morbidity and mortality rates (Seifert *et al.,* 1995; Cisneros *et al.,* 1996; Dijkshoorn *et al.,* 2007; Visca *et al,* 2011; Karakoc *et al.,* 2013).
In a nationwide study on infections occurring in patients admitted to the intensive care units (ICUs) of 45 Italian hospitals over the years 2002-2003, *A. baumannii* ranked third among the causative agents and all the isolated strains have shown a high level of resistance (58%) to all the antibiotics tested (Nicoletti *et al.,* 2006). Several other studies have shown the occurrence at hospital level of multi-drug resistant (MDR) and pandrug-resistant (PDR) *A. baumannii* isolates, including an increasing number of carbapenem-resistant strains (Poirel and Nordmann, 2006; Perez *et al.,* 2007, Souli *et al.,* 2008; Zarrilli *et al.,* 2009; Bahador *et al.,* 2013).
This resistance to carbapenems is most often mediated by oxacillinases (OXAs) and less frequently by metallo-β-lactamases (MBLs) (Zarrilli *et al.,* 2009; Cornaglia *et al.,* 2001; Azimi *et al.,* 2013).
The *A. baumannii* MDR phenotype seems to play an important role in the remarkable capacity of the microrganism to persist and spread in the hospital environment, together with its ability to colonize both biotic and abiotic surfaces and to grow as biofilm (Neely 2000; Villegas and Hartstein, 2003; Lee *et al.,* 2006; Rodriguez-Bano *et al.,* 2008; Zarrilli *et al.,* 2009; Eijkelkamp *et al.,* 2011; Gurung *et al.,* 2013). Because of the presence of dormant cells, uncommonly found in other Gram-negative bacteria, the environmental persistence of *A. baumannii* fits the reported ability of some clinical isolates to survive for a long time on abiotic surfaces under desiccated conditions (Wendt *et al.,* 1997; Gayoso *et al.,* 2013).

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Despite the large number of papers on the association between *A. baumannii* hospital outbreaks, severe infections and antibiotic resistance, factors determining the virulence and pathogenicity of this microorganism have to be further elucidated. However, it is becoming evident that biofilm-forming ability can be considered one of the main virulence factors common to a large number of *A. baumannii* clinical isolates (Rodriguez-Baño *et al.,* 2008; King *et al.,* 2009; Eijkelkamp *et al.,* 2011; Gurung *et al.,* 2013).

To date, biofilm-related virulence determinants of this emerging pathogen include the CsuA/BABCDE pilus usher-chaperone assembly system, regulated by a two-component system (BfmS/BfmR) (Tomaras *et al.,* 2003, Tomaras *et al.,* 2008; deBreij *et al.,* 2009), the outer membrane protein OmpA of 38 kDa (Choi *et al.,* 2005), the outer membrane protein of 854 kDa, with a high similarity to the staphylococcal biofilm-associated protein (Bap) (Lohefem *et al.,* 2008), the autoinducer synthase AbaI, part of the *quorum sensing* (QS) system (Niu *et al.,* 2008) and the *pgaABCD* operon responsible for the production of poly-β-1,6-*N*-acetylglucosamine (PNAG) (Choi *et al.,* 2009).

A recent study on gene expression in biofilm-growing *A. baumannii* cells, compared with the planktonic cells, showed in sessile cells a deep reorganization of amino acids and fatty acid metabolism, motility, active transport, DNA-methylation, iron acquisition, transcriptional regulation and QS. In fact, 1621 genes resulted over-expressed in biofilms, including 55 genes exclusively expressed in *A. baumannii* sessile cells (Rumbo-Feal *et al.,* 2013).

This review summarises the current state of knowledge on *A. baumannii* biofilm formation, its clinical significance and the factors involved in the process and in its regulation.

## ADHERENCE TO BIOTIC AND ABIOTIC SURFACES AND CLINICAL IMPLICATIONS

The ability of *A. baumannii* to adhere and form biofilm has been demonstrated on both biotic and abiotic surfaces.

Regarding the interaction of *A. baumannii* with host cells, a first scanning electron microscopy (SEM) study demonstrated that the pili-mediated *A. baumannii* adherence to epithelial cells has to be considered the initial step for colonization and subsequent host infection, even if the ability to adhere, a general feature of this species, varies among different *A. baumannii* clinical isolates (Lee *et al.,* 2006).

Recently evidence has shown that biofilm formation at the solid-liquid interface is at least three times higher in *A. baumannii* than in the other *Acinetobacter* species (80-91% versus 5-24%), giving rise to a thick pellicle clearly visible on the top of broth cultures (Martí *et al.,* 2011).

As far as adhesion on abiotic surfaces is concerned, numerous studies have revealed a high propensity of *A. baumannii* clinical isolates to form biofilm on different substrata, such as glass (Fig. 1) or plastic (Tomaras *et al.,* 2003; Eijkelkamp *et al.,* 2011).

This ability of *A. baumannii* to grow as biofilm on abiotic surfaces plays an important role in causing nosocomial infections, due to the surface colonization of hospital equipment and indwelling medical devices, such as urinary catheters, central venous catheters (CVCs), endotracheal tubes, etc. (Donlan, 2001; Trautner and Darouiche, 2004; Djeribi *et al.,* 2012). The interaction of different *A. baumannii* clinical strains with glass and plastic surfaces, hydrophilic and hydrophobic respectively, has been reported to vary according to the specific features of the different strains (McQueary and Actis, 2011).

With regard to urinary catheter colonization, device placement can trigger a catheter-associated urinary tract infection (CAUTI) in ICU patients because of the possible contamination, at insertion time, by one or more gram-positive or gram-negative bacterial species, thereby giving rise to a single- or, more often, multi-species biofilm. In fact, the ultrastructural analysis of the biofilm formed on the inner surface of catheter revealed a dense cell multilayer, formed by bacteria of different shapes and sizes, embedded in a rich esopolysaccharide matrix (Djeribi *et al.,* 2012).

The presence of multi-species biofilms on urinary catheter surfaces, as shown in Figure 2, was recently disclosed combining cultural methods and field emission scanning electron

microscopy (FESEM) observations (Donelli & Vuotto, 2014).

As regards CVC colonization, catheter-related bloodstream infections (CRBSIs) caused by gram-negative bacteria, albeit less common (14%) than those caused by the gram-positive bacteria (76%; Wisplinghoff *et al.*, 2003), have become increasingly frequent in the last decade and difficult to treat due to the rising incidence of antibiotic-resistant gram-negative bacilli, including *Acinetobacter* (Hanna 2004; Djeribi *et al.*, 2012).

*A. baumannii* has also been demonstrated to form biofilm on the inner surface of endotracheal tubes. In a prospective observational study performed in ICU patients mechanically ventilated for more than 24 hours, biofilm formation in 71 out of 75 collected tube specimens (95%) was reported, *A. baumannii* and *Pseudomonas aeruginosa* being the most frequent bacterial species isolated. Biofilm morphology and extension varied, depending on the tube specimen, from scarce and dispersed biofilms to mature biofilms, with an abundant matrix covering the entire surface of the observed samples (Gil-Perotin *et al.*, 2012).

## BIOFILM REGULATION

In all microorganisms, adhesiveness and biofilm formation are well-orchestrated processes responding to a wide range of cellular and environmental cues (Stanley and Lazazzera, 2004). This also applies to *A. baumannii* in which biofilm formation has been reported to be under the control of several factors, including the presence of antibiotic resistance genes, growth

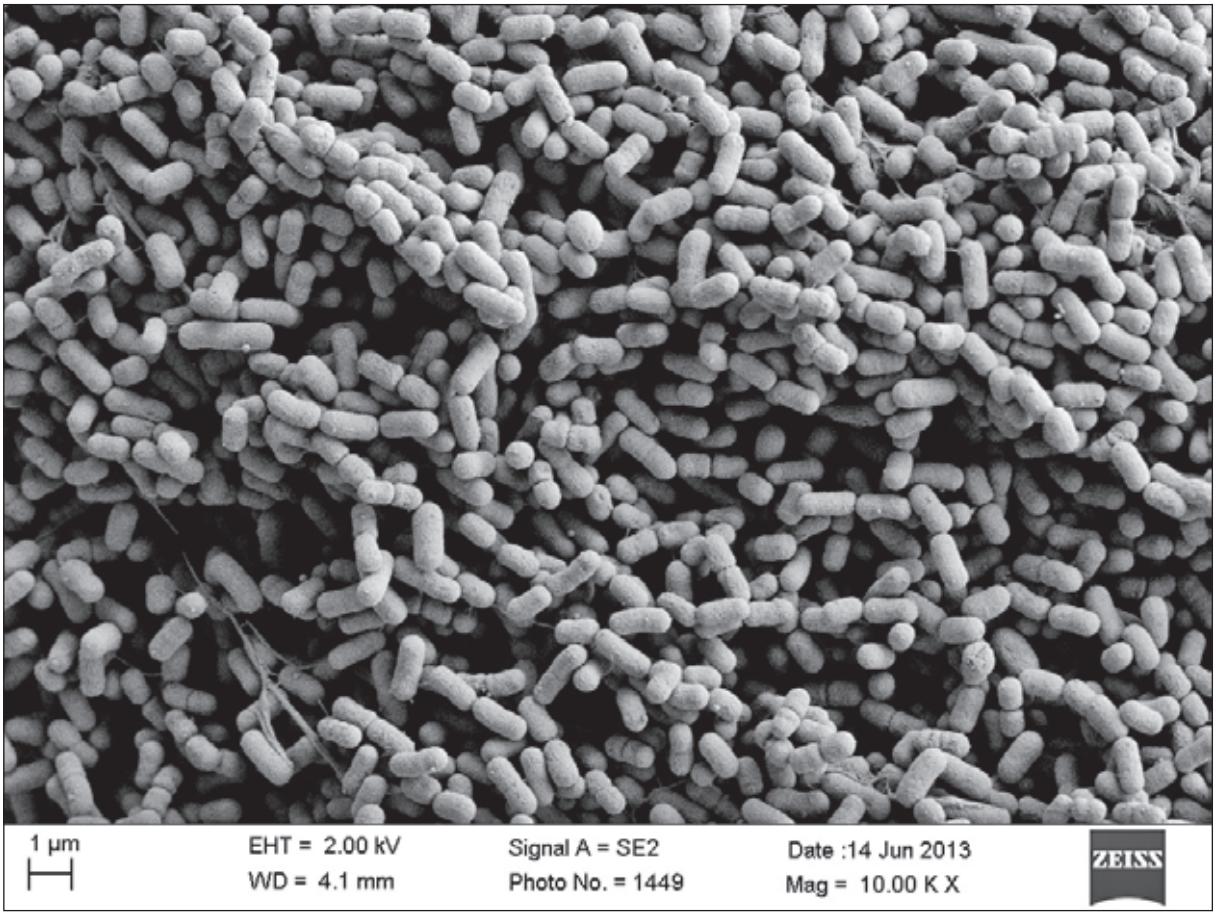


FIGURE 1 - *FESEM micrographs (magnification X 5,000) of* A. baumannii *in vitro biofilm formed on the surface of a glass coverslip after 24h of incubation at 37°C in Luria-Bertani broth.*

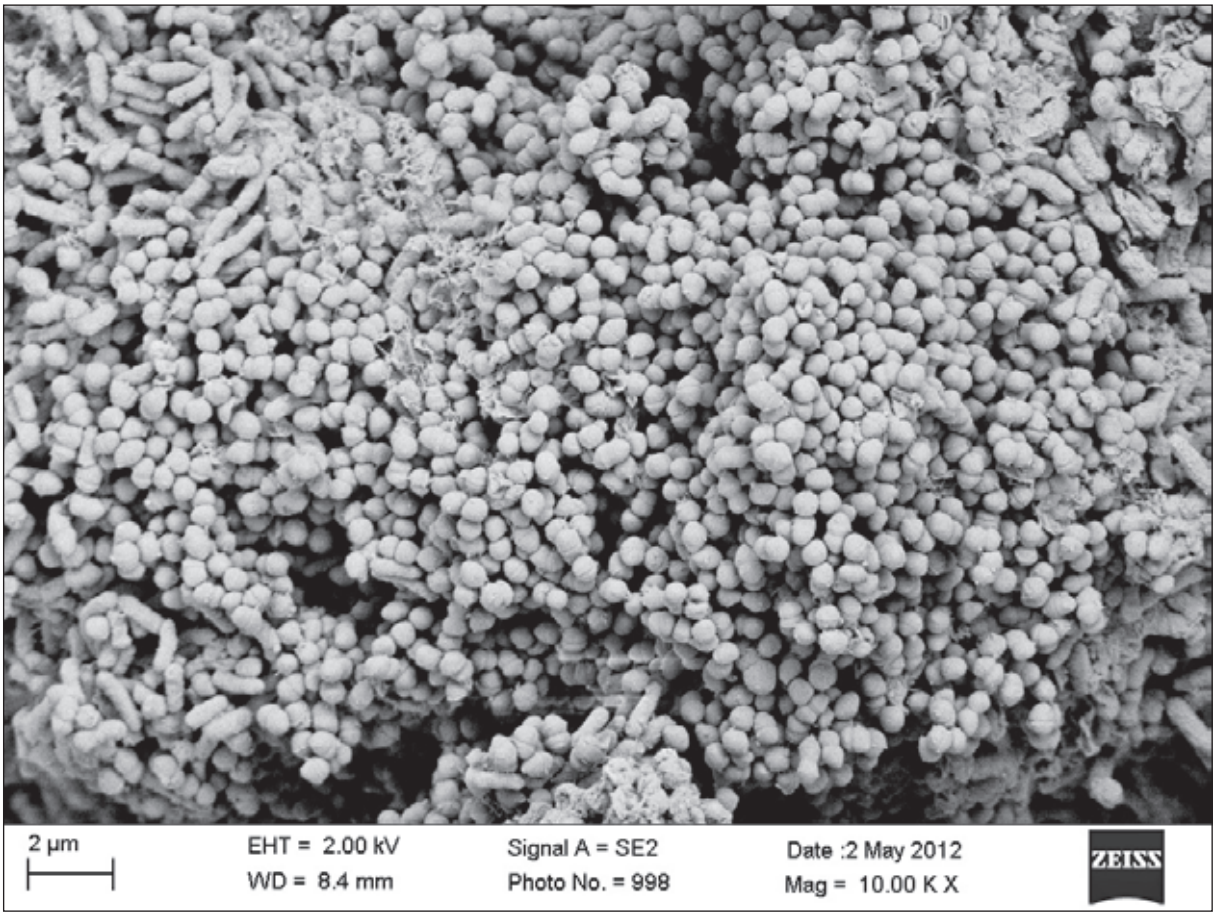


FIGURE 2 - *FESEM micrographs (magnification X 10,000) of a polymicrobial biofilm grown in the lumen of a Foley urinary catheter removed from a patient admitted to the Fondazione Santa Lucia research hospital for neuromotor rehabilitation in Rome. The species identified by culture methods were* Klebsiella pneumoniae, Pseudomonas aeruginosa *and* Acinetobacter baumannii.

conditions and cell density (Gaddy and Actis, 2009). In fact, Lee *et al.* (2008) have shown that adhesion of *A. baumannii* to both biotic surfaces, such as bronchial epithelial cells, and to plastic surfaces is enhanced by the presence and expression of the $bla_{PER-1}$ gene, even if the mechanism by which that occurs remains unsettled (Lee *et al.,* 2008). On the other hand, an independent study found that only 2 out of 11 human isolates carrying the $bla_{PER-1}$ gene are able to form a robust biofilm compared with isolates lacking this genetic determinant. Thus, these results bring to question the actual relevance of $bla_{PER-1}$ expression in biofilm formation (Rao *et al.,* 2008).

Environmental conditions such as the growth temperature and concentration of extracellular free iron, which are known to be relevant for *A. baumannii* host interaction, also affect the amount of biofilm formed on abiotic surfaces. Indeed, *A. baumannii* clinical isolates showed a significant reduction in adhesiveness and biofilm formation ability on biotic and abiotic surfaces (*i.e.* human respiratory epithelial cells and plastic, respectively) when grown in the presence of an iron-chelating agent (Lee *et al.,* 2006; 2008).

Interestingly, *A. baumannii* ATCC 17978 strain produced little or no biofilm on glass surfaces when it was incubated under blue light, while a normal biofilm was observed when cells were incubated in the darkness (Mussi *et al.,* 2010). This response is mediated by the BlsA photoreceptor protein, which contains a *N*-terminal

blue-light-sensing-using flavin domain. The mechanisms by which BlsA transduces the light signal and controls gene expression are not yet known. However, it has been demonstrated that the diverse transcription of *blsA* at 28°C and 37°C differentially affects the response of *A. baumannii* biofilm to the light. In fact, this response seems to have a global effect on *A. baumannii* physiology, affecting not only biofilm formation but also motility and virulence (Mussi *et al.,* 2010).

Recently, ethanol has also been reported to affect biofilm formation on abiotic surfaces. In fact, production of proteins involved in lipid and carbohydrate anabolism was shown to increase in the presence of ethanol, thereby increasing carbohydrate biofilm content and thus enhancing biofilm formation and decreasing bacterial motility (Nwugo *et al.,* 2012).

Both clinical and environmental isolates of *Acinetobacter spp*. were reported to produce QS signal molecules by which bacteria control adhesiveness and biofilm formation in response to cell population density (Gonzalez *et al.,* 2001; 2009; Bhargava *et al.,* 2010). The *A. baumannii* M2 clinical strain was demonstrated to produce an *N*-acyl-homoserine lactone (*i.e. N*-3-hydroxydodecanoyl-homoserine lactone), this QS molecule being important for the formation of biofilm on abiotic surfaces (Niu *et al.,* 2008).

On the other hand, it has recently been demonstrated that the homoserine lactone synthase (A1S_0109) of *A. baumannii* ATCC 17978 strain was over-expressed in biofilm-growing cells with respect to planktonic cells (Rumbo-Feal *et al.,* 2013).

Although the data presented so far offer an insight into the regulation of the biofilm formation process in *A. baumannii,* the different cellular and/or environmental signals to which this process responds await further clarification.

## FACTORS INVOLVED IN BIOFILM FORMATION

According to the multifactorial nature of biofilm formation, a number of gene products have been reported to play a role in adhesiveness and biofilm development of *A. baumannii* on both abiotic and biotic surfaces. However, there seems to be no close correlation between adhesion on host cells and biofilm formation on abiotic surfaces, a wide variability in cell-cell and cell-surface interactions being exhibited by different *A. baumannii* clinical isolates. This variability can be especially observed in the adherence on biotic or abiotic surfaces mediated by different pili-like structures. Early studies on the *A. baumannii* ATCC 19606T strain showed that pilus production mediated by the CsuA/BABCDE usher-chaperone assembly system is required for the initial steps of bacterial attachment on abiotic surfaces, resulting in microcolony formation followed by the full development of biofilm (Tomaras *et al.,* 2003). The operon *csuA*/*BABCDE* seems to be widespread among clinical isolates, the demonstrated ability of *A. baumannii* strain ATCC 19606T to adhere by pili to and to form biofilm on abiotic surfaces depending on the expression of *csuE*, which is part of the *csuA/BABCDE* usher-chaperone pili assembly system.

On the other hand, in a study on the involvement of CsuA/BABCDE-dependent pili in the interactions between *A. baumannii* 19606T and human bronchial epithelial cells, SEM investigations disclosed two types of cell appendages *i.e.* short pili and long extensions, the latter lacking the non-forming biofilm *csuE* isogenic insertion mutant and presumably being the CsuA/BABCDE-encoded extensions previously described. That study also showed that only the short and thin CsuA/BABCDE-independent pili are involved in bacterial adherence to human respiratory cells (de Breij *et al.,* 2009).

Furthermore, the expression of the *csuA*/*BABCDE* operon was found to be regulated by a two-component system constituted by the sensor kinase, encoded by *bfmS* and the response regulator encoded by *bfmR*. Insertional inactivation of *bfmR* resulted in a loss of expression of the *csuA/BABCDE* operon and the ensuing lack of pili production and biofilm formation on plastic when cells are cultured in rich medium. Inactivation of the *bfmS* sensor kinase gene resulted in a decrease, but not abolition of biofilm formation on abiotic surfaces also in *A. baumannii* ATCC 17978 strain (Tomaras *et al.,* 2008; Liou *et al.,* 2013), while it resulted in a loss of adherence to eukaryotic cells (Liou *et*

*al.,* 2013). Recently, the biochemical characterization of the BfmR protein was also elucidated showing a secondary structure prediction for the *C*-terminal domain, residues 130-238 (Olson *et al.,* 2012).

A different involvement of a global transcriptional repressor, a homologue of the histone-like nucleoid structuring (H-NS) protein, has also been observed in adhesion and biofilm formation on biotic and abiotic surfaces. This regulator represses *A. baumannii* motility, lethality toward *Caenorhabditis elegans* nematodes, adherence to human pneumocytes and biofilm formation at the air-liquid interface, but not the attachment to a polystyrene microtiter plate (Eijkelkamp *et al.,* 2013).

On the contrary, different proteins seem to play a key role in biofilm formation on both biotic and abiotic surfaces by promoting cell-surface and cell-to-cell adhesion. Literature data point out that the outer membrane protein, OmpA, a trimeric porin of 38 kDa acting as a general diffusion pore of size 1.3 nm, plays a role in the attachment step of *A. baumannii* on plastics, and also in the interaction of the pathogen with both human epithelial cells and *Candida albicans* filaments (Choi *et al.,* 2005; Choi *et al.,* 2008; Gaddy *et al.,* 2009). Other than its role in cell adhesion, OmpA is also a potential virulence factor, since it induces epithelial cell death, early-onset apoptosis and delayed-onset necrosis in dendritic cells, targeting the mitochondria and inducing the production of reactive oxygen species (Lee *et al.,* 2010).

The bacterial adhesin Bap (biofilm-associated protein), expressed on the cell surface and conserved among different clinical isolates, was first demonstrated by Loehfelm and coworkers (2008) in *A. baumannii* (307-0294 strain) to be involved in intercellular adhesion, thus ensuring biofilm maturation on different substrata (Loehfelm *et al.,* 2008; Goh *et al.,* 2013). SEM analyses of biofilm have shown that Bap is required for three-dimensional tower structure and water channel formation on medically relevant surfaces, including polypropylene, polystyrene, and titanium (Brossard and Campagnari, 2012). Moreover, the same study showed that Bap increases *A. baumannii* adherence to both normal human bronchial epithelial cells and normal human neonatal keratinocytes, probably by increasing the bacterial cell surface hydrophobicity.

A recent study looked for the presence of Bap in several *A. baumannii* strains and in different lineages, showing that the *bap* gene, approximately 16 kb in size, is highly prevalent (90% of the tested strains) and repetitive. The same showed that biofilm formation on abiotic surfaces by the Bap-positive strains could be inhibited by affinity-purified Bap antibodies, demonstrating the direct contribution of Bap to the biofilm growth of *A. baumannii* clinical isolates (Goh *et al.,* 2013).

In *A. baumannii* ATCC 17978 strain, a general *O*-linked protein glycosylation system has also been recently identified and demonstrated to greatly increase the biofilm-forming ability, emphasizing that glycosylation promotes the initial attachment and enhances mature biofilm mass and density.

These authors speculated that glycans of the glycoproteins may have a function in cell-to-cell adhesion. Interestingly, the *O*-glycosylation machinery appears to be present in all clinical isolates tested as well as in all of the genomes sequenced, suggesting the existence of a strong evolutionary pressure to retain this system (Iwashkiw *et al.,* 2012).

A more recent study on the same strain identified a different locus responsible for the synthesis of the *O*-pentasaccharide found on the glycoproteins. Mutagenesis of this locus *pglC* prevented the synthesis of both glycoproteins and capsule, resulting in abnormal biofilm structures, and attenuated virulence in mice. The Δ*pglC* mutant, similarly to the wild type strain, gave rise to dense aggregates on abiotic surfaces, while the analysis of the biofilm structure revealed a rough and disordered phenotype, suggesting a nonspecific attachment. Thus, this capsular polysaccharide seems to be implicated in the organization and structure of the biofilm (Lees-Miller *et al.,* 2013).

Biofilm development and maturation of *A. baumannii* clinical isolates also depend on the capacity to produce and secrete poly-β-1,6-*N*-acetylglucosamine (PNAG). This major component of the biofilm exopolysaccharidic matrix is encoded by a cluster of four genes (*pgaABCD*) and produced by almost all the clinical strains (Choi *et al.,* 2009).

## CONCLUSIONS

Adhesiveness and biofilm-forming ability in *A. baumannii* play a pivotal role in the host-pathogen interactions and in medical device-associated infections, involving a range of bacterial factors, multiple cell signals and environmental cues.

Currently, there seems to be no close correlation between the genetic determinants involved in adherence to eukaryotic cells and those implicated in the first step of biofilm formation on abiotic surfaces. However, considering the high variability among the clinical isolates, it should be taken into account that all the studies to date on the genetic determinants responsible for biofilm formation were performed in different *A. baumannii* strains.

Furthermore, additional investigations are needed on the correlation between *A. baumannii* ability to adhere and form biofilm, propensity to cause outbreaks and life-threatening infections and increasing antibiotic resistance. Indeed, the quantitative differences in biofilm formation among clinical isolates, in association with the epidemicity of strains and the severity of infections, have been poorly investigated so far and these important issues await further research in this field (Rao *et al.,* 2008; Wroblewska *et al.,* 2008; Gurung *et al.,* 2013).

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