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Regulation of *Acinetobacter baumannii* biofilm formation

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Abstract

Acinetobacter baumannii is a Gram-negative opportunistic nosocomial pathogen. This microorganism survives in hospital environments despite unfavorable conditions such as desiccation, nutrient starvation and antimicrobial treatments. It is hypothesized that its ability to persist in these environments, as well as its virulence, is a result of its capacity to form biofilms. *A. baumannii* forms biofilms on abiotic surfaces such as polystyrene and glass as well as biotic surfaces such as epithelial cells and fungal filaments. Pili assembly and production of the Bap surface-adhesion protein play a role in biofilm initiation and maturation after initial attachment to abiotic surfaces. Furthermore, the adhesion and biofilm phenotypes of some clinical isolates seem to be related to the presence of broad-spectrum antibiotic resistance. The regulation of the formation and development of these biofilms is as diverse as the surfaces on which this bacterium persists and as the cellular components that participate in this programmed multistep process. The regulatory processes associated with biofilm formation include sensing of bacterial cell density, the presence of different nutrients and the concentration of free cations available to bacterial cells. Some of these extracellular signals may be sensed by two-component regulatory systems such as BfmRS. This transcriptional regulatory system activates the expression of the usher-chaperone assembly system responsible for the production of pili, needed for cell attachment and biofilm formation on polystyrene surfaces. However, such a system is not required for biofilm formation on abiotic surfaces when cells are cultured in chemically defined media. Interestingly, the BfmRS system also controls cell morphology under particular culture conditions.

Keywords

abiotic surface; *Acinetobacter*; biofilm; biotic surface; pili production; quorum sensing; signal transduction; transcriptional regulation

The genus *Acinetobacter* is a genetically diverse group of aerobic Gram-negative nonfermenting bacteria [1]. Although acinetobacters are commonly described as being ubiquitous in nature, those strains belonging to the *Acinetobacter baumannii–Acinetobacter calcoaceticus* cluster are emerging as problematic opportunistic pathogens due to the rapid increase in multidrug or pandrug resistance [2–4]. These infections manifest as serious diseases

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in compromised human hosts particularly in the case of ventilator-acquired pneumonia, urinary tract infections, septicemia, wound infections and more recently in severe cases of necrotizing fasciitis [5]. Furthermore, these infections have recently come under scrutiny because of their prevalence in wounded American military personnel returning from the Middle East [6]. Owing to the increase in mortality as well as the lack of treatment options for these infections, prevention of host colonization is absolutely necessary [7]. In addition, since a large proportion of these infections are acquired in healthcare environments, a better understanding of how this microorganism survives and persists in this environment is essential. It is hypothesized that the ability of clinical strains to survive desiccation, antimicrobial therapies and nutrient availability stress is mediated by the microorganism's ability to form biofilms on medically relevant surfaces.

Biofilm structure & function

A. baumannii clinical isolates have been observed to possess the ability to survive long stretches of time under highly desiccated conditions on abiotic surfaces [8,9]. For instance, Jawad and colleagues discovered that the mean survival time for a bank of 39 clinical isolates when subjected to desiccation on glass coverslips was 27 days, a response that is more common among Gram-positive than Gram-negative bacteria [8]. Accordingly, analysis of a collection of clinical isolates showed that the ability to form biofilms on abiotic surfaces is a common trait among different *A. baumannii* strains, particularly in those isolated from catheter-related urinary tract or bloodstream infections as well as a case of shunt-related meningitis [10]. In addition, *A baumannii* clinical isolates have shown a correlation between antibiotic resistance and the ability to adhere to clinically relevant surfaces including polystyrene and respiratory epithelial cells [11]. It was observed that cell adhesiveness and biofilm formation on plastic is higher in strains harboring the *blaPER-1* gene than in those that do not harbor this genetic trait. Furthermore, the level of expression of this gene, as determined by reverse transcription-PCR, is positively correlated with the level of biofilm formed on plastic and the adhesiveness of bacteria to human epithelial cells [11]. Similar findings were reported by Rao *et al.*, who found a significant association between multidrug resistance and biofilms, although they believe that the presence of *blaPER-1* is more critical for cell adhesion than the formation of bacterial biofilms on abiotic surfaces [12]. Thus, the functions of the biofilm structures formed by this bacterial pathogen encompass its ability to resist antimicrobial therapies as well as other environmental stresses such as dehydration and limited nutrient availability.

A biofilm is a community of multiple bacterial cells associated with a surface (either biotic or abiotic), arranged in a tertiary structure in intimate contact with each other and encased in an extracellular matrix that can be comprised of carbohydrates, nucleic acids, proteins and other macromolecules [13]. Furthermore, this structure can confer resistance to antimicrobial therapies on the order of one thousand times greater than that of their planktonic counterparts [14]. Bacterial biofilm initiation and development is not simply a serendipitous adherence of bacterial cells to a surface. On the contrary, it is a highly regulated series of molecular events, which cells keep under tight regulation. The most common factors that can influence biofilm formation are: nutrient availability, bacterial appendages (pili and flagella), bacterial surface components (outer membrane proteins, adhesins), quorum sensing and macromolecular secretions (polysaccharides, nucleic acids and so on) [15]. In addition, complex regulatory networks including two-component regulatory systems and transcriptional regulators are known to be responsible for the expression of a variety of biofilm-associated gene products in response to a wide range of environmental signals [16].

Acinetobacter baumannii biofilm formation & regulation

The *A. baumannii* 19606-type strain forms biofilms on abiotic surfaces such as glass and polystyrene [17]. With respect to the medically relevant surface polystyrene, a polymer that is commonly used in the manufacturing of a variety of medical devices, the production of pili is essential for biofilm formation by this clinical strain. These pili are the product of the *csuA/BABCDE* six open-reading frame polycistronic operon. Comparative analysis showed that operons similar to those described in the 19606-type strain are also found in the genome of strains AB0056 (GenBank accession number NC_011586) [101], AYE [18] and 17978 [19]. Interestingly, the two latter strains also contain three additional loci that code for secretion functions potentially associated with pili assembly and adhesion [101]. A locus coding for a putative chaperon-usher secretion system was also found in the ACICU strain, although the number of genes and their order seems to be different from that described for the 19606 strain [20]. By contrast, no similar *csu* loci were located in the genome of *A. baumannii* SDF and *Acinetobacter baylyi* ADP1 [18].

Inactivation of *csuE*, which codes for the putative tip adhesin, results in the abolition of pili production as well as biofilm formation [17]. This observation suggests that CsuA/ BABCDE-mediated pili play a role in the initial steps of biofilm formation by allowing bacterial cells to adhere to abiotic surfaces and initiate the formation of microcolonies that precede the full development of biofilm structures. The expression of this operon is regulated by a two-component regulatory system comprised of a sensor kinase encoded by *bfmS* and a response regulator encoded by *bfmR*. Transcriptional and translational analyses show that the inactivation of bfmR results in a loss of expression of the *csu* operon and the consequent abolition of both pili production and biofilm formation on plastic when cells are cultured in rich medium [21]. However, inactivation of the open-reading frame encoding the BfmS sensor kinase results in an attenuation but not abolition of biofilm formation. This indicates that there could be cross-talk between other sensing components and the BfmR response regulator, a hypothesis that suggests that multiple and different environmental stimuli could control biofilm formation via the BfmRS regulatory pathway. Interestingly, the function of this regulatory system is not confined to biofilm initiation, but is also involved in cell morphology. Equally interesting is the fact that the composition of the culture medium and the interaction of cells with abiotic surfaces play a significant role when the BfmRS system is not expressed [21]. Taken together, these observations underscore the importance of extracellular signals, not only in biofilm initiation and development but also in the morphology of cells interacting with abiotic surfaces.

In the case of glass surfaces, transposon inactivation of a homolog of the staphylococcal biofilm-associated protein (Bap) in *A. baumannii* strain 307-0294 resulted in a destabilization of the mature biofilm [22]. This destabilization was measured as a reduction of both biofilm volume and thickness upon imaging of cells producing green fluorescent protein under confocal laser-scanning microscopy. Bap is a protein exposed on the surface of the bacterial cell, as was evidenced by flow-cytometry analysis of immunofluores-cent-labeled whole bacterial cells. Based on this cellular location and its participation in biofilm development, it is hypothesized that this protein is involved in cell–cell interactions that support biofilm maturation. Furthermore, this protein is conserved among a panel of 98 *Acinetobacter* strains [22], a finding that underscores the importance of this protein in adhesion and biofilm formation. However, at present, there are no reports describing the potential environmental factors and conditions that could control the differential expression of the *bap* gene.

Another factor that has been observed to control the formation of biofilms by *A. baumannii* is the presence of metal cations. The *A. baumannii* 19606-type strain forms more biofilms on plastic when cultured in a chemically-defined medium under iron-chelated conditions, which

were imposed by the presence of the synthetic iron chelators 2,2′-dipyridyl and ethylenediamine-di-(o-hydroxyphenyl) acetic acid [17]. Such a response is in line with the well-known role that iron in particular, and metals in general, play in the differential expression of bacterial genes, some of which are central to the virulence of relevant pathogenic microorganisms. The analysis of a bank of multidrug-resistant *A. baumannii* clinical isolates also showed that the presence of the chelating agent ethylene-diaminetetraacetic acid caused a significant reduction in bacterial attachment and biofilm formation on human respiratory epithelial cells as well as on plastic surfaces [11]. The molecular mechanism by which these two cations, particularly in the case of iron where the Fur repressor proteins plays a central role in gene expression, remains to be elucidated.

Another pathway by which *A. baumannii* senses extracellular signals and directs biofilm formation is quorum sensing. The *A. baumannii* M2 strain produces an acyl-homoserine lactone molecule, a product of the *abaI* autoinducer synthase gene, the expression of which is positively upregulated by a feedback loop [23]. Mutagenesis of the *abaI* gene showed that this secreted product plays a role in the later stages of biofilm formation. Its inactivation results in a 30–40% biofilm reduction when compared with the isogenic parental strain. Exogenous addition of purified M2 acyl-homoserine lactone restored biofilm maturation of the *abaI* mutant. Thus, communication between bacterial cells with respect to cell density is integral to the maturation of *A. baumannii* biofilm as described for other relevant human bacterial pathogens [24].

In addition to abiotic surfaces, *A. baumannii* has evolved sophisticated methods to attach to biotic surfaces such as epithelial cells [11]. Although the mechanisms of attachment to human bronchial epithelial cells remain obscure, there is a positive correlation between the level of expression of the *blaPER-1* broad-range β-lactamase gene and the level of bacterial adhesion and biofilm formation on this type of cell. Such a correlation may explain the ability of *A. baumannii* to persist and disseminate in medical environments and the human host even in the presence of a wide range of antibiotics.

Our preliminary work has also shown that the *A. baumannii* 19606-type strain attaches to, and forms biofilms on, human alveolar epithelial cells and *Candida albicans* filaments but not yeast cells (Figure 1) [25]. This process does not require the expression of the *CsuA*/*BABCDE* operon since an isogenic derivative with a disruption in the *csuE* coding region showed the same biofilm phenotype as the parental strain. This is in contrast to the observations described earlier in which the expression of this gene and the subsequent assembly of pili are essential steps for biofilm formation on polystyrene. Equally contrasting is the recent observation that the *A. baumannii* 19606 *CsuE* mutant is able to adhere to human respiratory epithelial cells [26]. Based on this information and the role BfmRS plays in biofilm formation on abiotic surfaces, it is tempting to speculate that this two-component regulatory system may not play a role in the interaction of *A. baumannii* with a biotic surface such as that of *C. albicans* filaments. However, the extent and components of the BfmRS regulon are still unknown and it is possible that this regulatory system does infact play a role when bacterial and eukaryotic cells interact independently of its Csu-regulatory functions. It is interesting to note that a recent report also examined the interaction of *A. baumannii* with *C. albicans* although in a different experimental context since it included the nematode *Caenorhabditis elegans* [27]. This work, which does not provide direct evidence of bacterial attachment to, and biofilm formation on, fungal filaments, observes that inactivation of a homolog of the *gacS* sensor kinase attenuates the virulence of *A. baumannii* when tested using the tri-partite *C. elegans–A. baumannii–C. albicans* experimental model. Taken together, these observations suggest that biofilm formation on fungal filaments and their killing by *A. baumannii* are processes that are controlled by different regulatory systems that may respond to different environmental cues.

Conclusion & future perspective

The ability of *A. baumannii* to form biofilms is multifactorial and diverse, dependant upon the surface with which the cells are interacting. The expression of bacterial-associated factors in biofilm development is dependent upon nutrients and sensing of the environment by either the BfmS sensor kinase, cross-talk with other kinases or substrate-level phosphorylation of the cognate response regulators such as BfmR. In addition to these factors, surface proteins such as a Bap homolog could be involved in stabilizing the mature biofilm on abiotic or biotic surfaces. The presence of metal cations and the expression of resistance to broad-spectrum antibiotics can also increase the ability of *A. baumannii* to adhere to, and form biofilms on, a surface. However, many of the molecular mechanisms by which these bacteria adhere to diverse, medically relevant surfaces and human host cells remain obscure. Elucidating these mechanisms using modern and global approaches could provide missing basic information on these processes, which could be novel targets for future antimicrobial strategies as the age of antibiotics begins to wane. These are realistic and achievable goals since *A. baumannii* has entered the genomic and postgenomic era after several genomes were fully sequenced and annotated or are close to completion. Comparative genomics has already shed light on the common and unique genetic features of different clinical isolates, such as the presence of a unique resistance island in a multidrug-resistant nosocomial isolate [28]. These advances, together with the possibility of conducting global gene-expression analyses and testing virulence with appropriate experimental models, should provide a quantum leap in our understanding of not only biofilm-formation functions but also how these functions correlate with other cellular factors that contribute to the virulence of *A. baumannii* and its ability to cause severe infections in humans.

Executive summary

- Acinetobacter baumannii strains have emerged as problematic pathogens due to multi- and pandrug resistance, making treatment difficult.
- Prevention of colonization and persistence is key, thus a better understanding of microbial survival in healthcare settings could facilitate lower colonization rates.
- This survivability characteristic of *Acinetobacter* is hypothesized to be mediated by biofilm formation in the healthcare setting.

Biofilm structure & function

- Biofilms are structural arrangements of cells on a surface that are encased in an extracellular matrix. These structures can confer resistance to antimicrobial compounds.
- *A. baumannii* clinical isolates survive following long periods of desiccation and form biofilms on abiotic and biotic surfaces.
- Multidrug resistance correlates with the ability to attach to polystyrene and epithelial cells. Furthermore, expression of *blaPER-1* correlates with cell adherence and biofilm formation.
- Regulatory networks tightly control the expression of biofilm-associated factors such as cellular appendages, adhesions and cell density-sensing molecules.

Acinetobacter baumannii biofilm formation & regulation

- The 19606-type strain forms biofilms on polystyrene. The initiation of these structures is mediated by somatic pili produced by the CsuA/BABCDE chaperone-

usher secretion system, the expression of which is regulated by the two-component system BfmRS.

- A homolog of staphylococcal biofilm-associated protein has been identified as a surface component of *A baumannii* that is involved in biofilm stabilization and maturation.
- Several environmental signals have been identified that influence biofilm formation including the presence of metal cations and quorum-sensing molecules.
- The 19606-type strain attaches to, and forms biofilms on, biotic surfaces such as *Candida albicans* filaments and human alveolar epithelial cells. These interactions result in the killing of the eukaryotic cells.

Conclusion & future perspective

- The regulation of biofilm formation is multifactorial including a variety of environmental signals and sensing molecules that result in the production of a variety of bacterial factors, including surface proteins and pili that play a role in adhesion and biofilm development.
- Although *A. baumannii* has been observed to form biofilms on both abiotic and biotic surfaces, many of the molecular mechanisms remain unidentified.
- The advent of sequenced and annotated genomes for clinically relevant strains provides excellent resources for the investigation of genes and gene products involved in these processes.
- Global gene-expression analysis and animal model experiments can shed light on the virulence properties of this pathogen.

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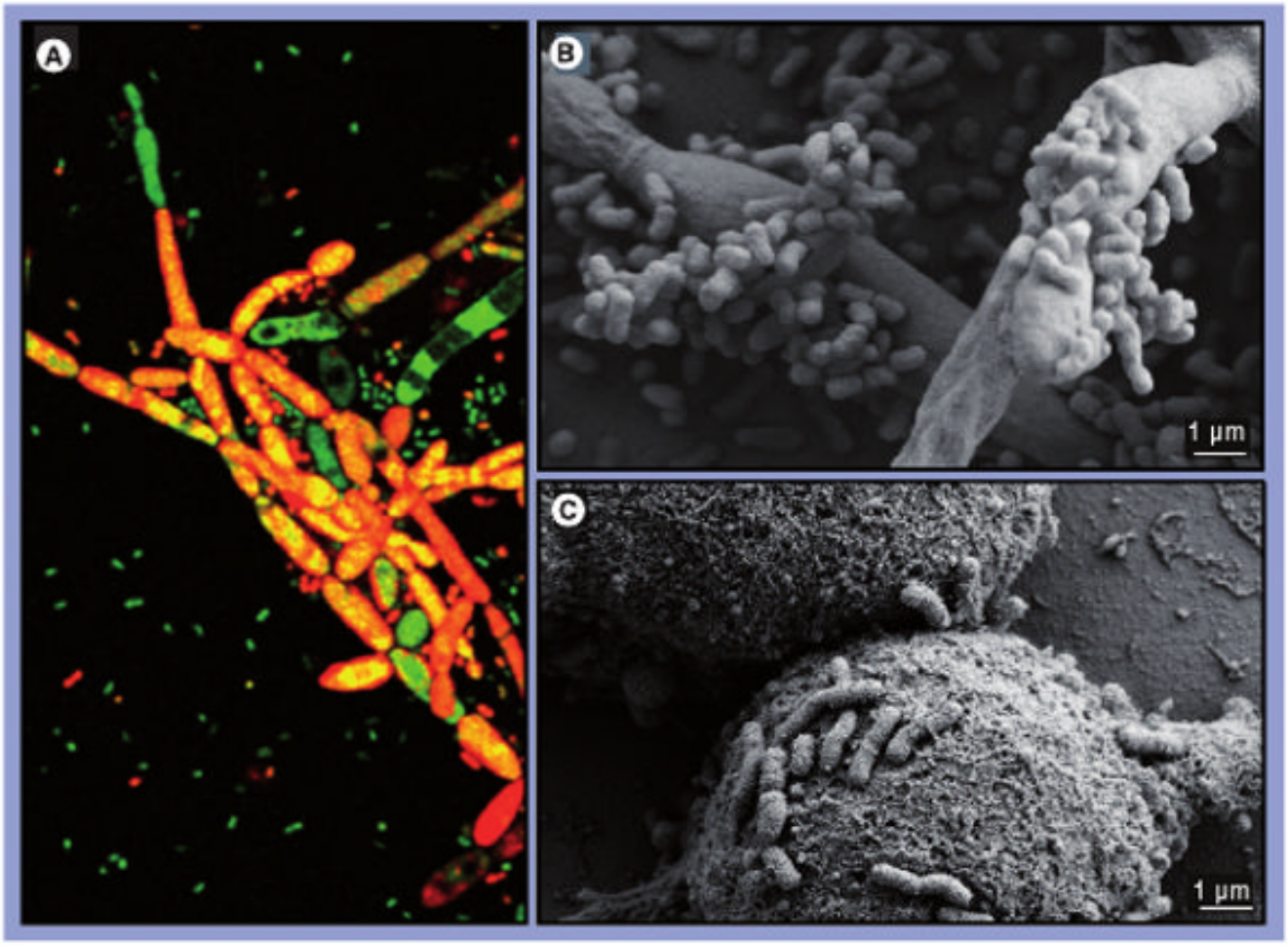

Figure 1. Microscopy analysis of *Acinetobacter baumannii* 19606 cells attached to eukaryotic cells
(A) Laser-scanning confocal microscopy of live/dead-stained *A. baumannii* 19606 cells attached to *Candida albicans tup1* filaments. Live bacterial cells, stained green, attached to the surface of dead fungal filaments, stained red, appear as areas of yellow co-fluorescence. The micrograph was taken at 400× magnification. **(B)** Scanning electron microscopy (SEM) of bacterial cells attached to *C. albicans tup1* filaments. **(C)** SEM of bacterial cells attached to A549 human alveolar epithelial cells.