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Uncovering the mechanisms of *Acinetobacter baumannii* virulence

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Abstract

Acinetobacter baumannii is a nosocomial pathogen that causes ventilator-associated as well as bloodstream infections in critically ill patients and the spread of multidrug-resistant (MDR) *Acinetobacter* strains is cause for concern. Much of the success of *A. baumannii* can be directly attributed to its plastic genome, which rapidly mutates when faced with adversity and stress. However, fundamental virulence mechanisms, beyond canonical drug resistance, were recently uncovered that enable *A. baumannii*, and to a limited extent other medically relevant *Acinetobacter* species, to successfully thrive in the healthcare environment. In this Review, we explore the molecular features that promote environmental persistence, including desiccation resistance, biofilm formation and motility, and we discuss the most recently identified virulence factors, such as secretion systems, surface glycoconjugates and micronutrient acquisition systems, that collectively enable these pathogens to successfully infect their hosts.

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Recently, virulence mechanisms, beyond canonical drug resistance, were uncovered that enable *Acinetobacter baumannii* to thrive in the healthcare environment and cause infections in critically ill patients. Feldman *et al.* explore the molecular features that promote environmental persistence and the most recently identified virulence factors that enable successful infection of the hosts.

Keywords

Biological sciences; Microbiology; Bacteriology; Pathogens; Bacteria; Bacterial pathogenesis; Bacterial physiology; Bacterial secretion; Antibacterial drug resistance

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Further Reading:

List of Prokaryotic names with Standing in Nomenclature: <http://www.bacterio.net/acetobacter.html>

Acinetobacter baumannii is an opportunistic human pathogen that predominantly infects critically ill patients. Once thought to be benign, *A. baumannii* is now considered a global threat in the healthcare setting mainly owing to its propensity to acquire multidrug, extensively drug and even pandrug resistance phenotypes at previously unforeseen rates^{1,2}.

Infections caused by *A. baumannii* account for ~2% of all health care-associated infections in the United States³ and Europe⁴; however, these rates are twice as high in Asia and the Middle East⁴. Although infection rates are lower compared with other Gram-negative pathogens, globally, ~45% of all isolates are considered to be multidrug resistant (MDR), with rates as high as 70% in Latin America and the Middle East¹. These daunting MDR rates are nearly four times higher than those observed for other Gram-negative pathogens, such as MDR *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, for which global surveillance statistics are also available¹. In light of this, the Centers for Disease Control and Prevention (CDC) categorized MDR *Acinetobacter* as a serious threat, thus prompting continual public health monitoring and prevention activities⁵. Furthermore, the World Health Organization (WHO) has included carbapenem-resistant *A. baumannii* in the critical group in the list of bacteria that pose the greatest threat to human health, prioritizing research and development efforts for new antimicrobial treatments⁶.

A. baumannii causes a range of nosocomial infections across multiple anatomical sites⁷. Most commonly, *A. baumannii* infections manifest as ventilator-associated pneumonia or central line-associated blood stream infections⁸. Less frequently, *A. baumannii* causes infections in the skin and soft tissues and at surgical sites as well as catheter-associated urinary tract infections^{8,9}. Common to each of these scenarios is a breach in an anatomical barrier that enables the entry of *A. baumannii* directly to the site of infection. Community-acquired infections caused by *A. baumannii* have also been reported¹⁰. To date though, community-acquired infections have only presented in patients with underlying co-morbidities such as alcoholism, diabetes mellitus or other illnesses such as cancer and obstructive pulmonary disorders¹⁰.

Currently, there are more than 50 designated *Acinetobacter* species (List of Prokaryotic names with Standing in Nomenclature), of which the overwhelming majority are considered non-pathogenic; however, as mentioned above, a select few species are opportunistic human pathogens. The most clinically relevant members of the *Acinetobacter* genus phylogenetically cluster into the *Acinetobacter calcoaceticus-baumannii* (*Acb*) complex¹¹. The *Acb* complex consists of five pathogenic species, *A. baumannii*¹², *Acinetobacter nosocomialis*¹³, *Acinetobacter pittii*¹³, *Acinetobacter seifertii*¹⁴ and *Acinetobacter dijkschoorniae*¹⁵, as well as one non-pathogenic species, *Acinetobacter calcoaceticus*. The most clinically relevant and well-characterized *Acinetobacter* species is *A. baumannii*. This can partially be attributed to the inability to phenotypically distinguish *A. baumannii* from other members of the *Acb* complex, and until recently this has hindered appropriate species identification. However, the use of matrix-associated laser desorption ionization-time of flight (MALDI-ToF) mass spectrometry to identify species-specific outer membrane components of each member of the *Acb* complex has greatly enhanced species identification^{7,16}. Nevertheless, given that all five pathogenic members of the *Acb* complex are frequently identified as *A. baumannii*, the designation of *A. baumannii*, unless otherwise

stated, will be used in the broad sense to encompass all pathogenic members of the *Acb* complex.

The genetic relatedness between members of the *Acb* complex and their phenotypical similarities might indicate that they share common virulence factors, rendering studies in *A. baumannii* potentially applicable to other pathogenic *Acinetobacter* species. Indeed, some clinically relevant and recently described virulence attributes of pathogenic *Acinetobacter* species were first described in *A. nosocomialis* and subsequently characterized in *A. baumannii* (discussed below). This is particularly relevant as the number of scientific studies focusing on the pathobiology of *Acinetobacter* species overwhelmingly use *A. baumannii* as the model organism. However, we are now starting to observe discrete clinical and phenotypic characteristics between members *Acb* complex, particularly, *A. baumannii*, *A. pittii* and *A. nosocomialis*¹⁷.

Over the past decade, we have begun to unravel the exceptional and complex mechanisms that led to the emergence of *A. baumannii* as a formidable human pathogen, particularly beyond the canonical drug resistance mechanisms that have been extensively studied. Although many common features emerge, there is a clear absence of any discernable toxin or molecular determinant that can account for the virulence potential of a particular *A. baumannii* strain. Instead, our current understanding of *A. baumannii* virulence suggests a ‘persist and resist’ strategy. Specifically, *A. baumannii* has a remarkable capacity to survive in unfavorable conditions. In this Review, we discuss our current understanding of the virulence mechanisms in *A. baumannii*. Particularly, we explore the molecular features that promote environmental persistence, including desiccation resistance, biofilm formation and motility, and we discuss the most recently identified virulence factors, such as secretion systems, surface glycoconjugates and micronutrient acquisition systems, that facilitate *A. baumannii* pathogenesis. For a comprehensive review regarding the clinical and pathophysiological traits of *A. baumannii*, the reader is referred to a recently written article by experts in the field¹⁸.

Environmental persistence

It is largely believed that two attributes, drug resistance and environmental persistence, have enabled *A. baumannii* to thrive in the nosocomial environment¹⁹. Below, we discuss those features that enable *A. baumannii* to persist in environments that are inhospitable to many bacterial pathogens, thus, setting the stage for human colonization and subsequent infection.

Disinfection, desiccation and oxidative stress resistance mechanisms.

Commonly, healthcare environments include prolonged periods of desiccation and routine disinfection regimes. Similar to antibiotic resistance, *A. baumannii* has adapted to those stresses¹⁸.

Desiccation resistance, which is the ability to maintain viability under dry conditions, varies amongst clinical isolates of *A. baumannii*, with some isolates remaining viable for almost 100 days^{20,21}. Desiccation resistance in *A. baumannii* is multi-factorial and not yet fully defined. However, it is clear that desiccation resistance depends on the ability of *A.*

baumannii to maintain viability under conditions of water limitations. Indeed, in *Acinetobacter baylyi*, a non-pathogenic relative of *A. baumannii*, capsular polysaccharides (which are composed of repeating carbohydrate units and function as a glycan shield encompassing the entire bacterium and protecting from external threats) promotes survival during periods of desiccation²². Although direct evidence is lacking, given the similar biosynthetic pathways for capsular polysaccharide in *A. baylyi* and *A. baumannii*²³, the ability of capsule [G] to retain water in *A. baumannii* and the presence of a capsular polysaccharide covering *A. baumannii* cells grown in a biofilm under dry conditions²⁴, it is likely that the capsule contributes to resistance to desiccation in *A. baumannii*. Furthermore, a recent study has linked desiccation resistance to the composition of the outer membrane²⁵. Specifically, a mutant strain that produces under-acylated lipooligosaccharide [G] (LOS) was unable to survive periods of desiccation. The authors suggested that the increased membrane fluidity resulting from the altered lipid composition of the outer membrane in this mutant would likely permit leakage of water and hydrophilic nutrients out of the cell²⁵. Remarkably, a recent study found that the total bacterial counts and total culturable counts of *A. baumannii* did not change during prolonged dry periods, which indicates that transitioning to a dormant state, defined by a significant portion of the population entering a viable but non-culturable state, is not a major strategy for *A. baumannii* to persevere in desiccated environments²⁶. However, the mechanisms behind desiccation persistence were not investigated and remain to be fully characterized.

Aside from the obvious loss of water during periods of desiccation, desiccation-rehydration causes various DNA lesions, including alkylation, oxidation, cross-linking, base removal and strand breaks²⁷. To help prevent some of the DNA damage induced by desiccation-rehydration, *A. baumannii* relies on the protective role of the RecA protein²⁸, which is an enzyme that is required for homologous recombination and recombination repair. A study showed an ~50 fold increase in the mutation frequency during a round of desiccation and rehydration of *A. baumannii*, as measured by the spontaneous appearance of rifampicin-resistant colonies²⁹. This finding leads to the provocative hypothesis that resistance to desiccation may contribute to the MDR phenotype of *A. baumannii*.

Moreover, oxidative stress is also induced under periods of desiccation. As a result, *A. baumannii* significantly upregulates proteins that are associated with detoxifying reactive oxygen species³⁰. Some *Acinetobacter* spp. are believed to have the highest tolerance to hydrogen peroxide outside of spore forming Gram-positive bacteria. A strain of *Acinetobacter gyllenbergii* is able to withstand 100mM hydrogen peroxide with no loss in viability and even maintain viability in 320mM hydrogen peroxide³¹. As *A. gyllenbergii* has been isolated from human specimens³², it is likely that more clinically relevant *Acinetobacter* spp., such as *A. baumannii*, will develop extremotolerances towards oxidative stressors given the genomic plasticity in members of the *Acb* complex. In fact, in response to oxidative stress, the emergence of *A. baumannii* strains that contain the insertion sequence element, IS*Aba1*, upstream of the catalase [G] gene, *katG*, has been reported, which drives the expression of *katG* and enhances resistance to increased levels of hydrogen peroxide³³. However, more experimental evidence is warranted.

Disinfectants such as chlorhexidine are extensively used in hospitals and other health care settings. Chlorhexidine, which is an antiseptic that is effective against Gram-negative and Gram-positive bacteria, disrupts cell membranes. *A. baumannii* has been shown to actively pump chlorhexidine out of the cell using the *Acinetobacter* chlorhexidine (AceI) efflux protein³⁴, thus possibly promoting survival of the bacteria under stress conditions. Another stressor, ethanol, has been shown to promote the growth and virulence of *A. baumannii*^{35,36}; albeit, at low concentrations³⁷. Moreover, physiological concentrations of ethanol found in the blood streams of individuals with a history of alcohol use disorder sufficiently impair phagocytosis [G] and thus elimination of *A. baumannii*³⁸. Expectedly, chronic alcohol consumption is one of the primary risk factors associated with community-acquired *A. baumannii* infections¹⁰.

Although the role of environmental persistence has generally been accepted to be a virulence strategy of *A. baumannii*, much work is needed to determine the full underlying molecular mechanism.

Biofilm formation and maintenance.

Microbial biofilms, which are communities that are encased in an extracellular matrix, are produced by many if not all bacteria. Biofilms are likely to have an important role in the interactions of *A. baumannii* with its host, and biofilm formation contributes to medical device-associated infections.

A. baumannii populations within skin and soft tissue infections form robust biofilms, both within the wound and on occlusive dressings³⁹. *A. baumannii* also form biofilm communities on most abiotic surfaces; including, health care-associated equipment such as endotracheal tubes as well as polycarbonate and stainless steel⁴⁰. It is well regarded that bacteria within biofilm communities, including *A. baumannii*, have increased tolerance to extracellular stresses^{40,41}. As with many biofilm producing organisms, *A. baumannii* surface appendages, adhesins and protective surface structures, such as capsular polysaccharides, greatly contribute to the formation and maintenance of biofilms.

Although factors that contribute to biofilm formation seem to be strain-dependent, some common factors have been identified. Most *A. baumannii* strains encode for and produce a type I chaperone-usher pilus system designated Csu pili (Fig. 1A). Csu pili, regulated by the BfmRS two component regulatory system [G]⁴², are crucial for biofilm formation and maintenance on abiotic surfaces, including polystyrene⁴³, but, are not required for association with biotic surfaces such as human epithelial cells⁴⁴. Interestingly, most *A. baumannii* strains seem to carry the *csuA/BABCDE* locus; however, a subset of clinical isolates have lost the *csu* cluster⁴⁵, which indicates that these pili may not be required for biofilm formation and maintenance in all strains or that other pili systems may functionally replace them (see below). Moreover, a second two-component system termed GacSA⁴⁶, has been shown to moderately control *csu* gene expression and thus indirectly biofilm formation. Interestingly, sub-inhibitory concentrations of trimethoprim-sulfamethoxazole have been shown to completely repress the expression of Csu pili in *A. baumannii*, which indicates that improper use of antibiotics can alter population level behaviors and may promote a planktonic lifestyle⁴⁷. Other putative chaperone usher pili systems and Pap pili systems,

which are homologous to the P pili of *Escherichia coli*, have been implicated in *A. baumannii* biofilm formation and maintenance; yet, a detailed molecular analysis describing their specific role is lacking^{48,49}.

A. baumannii also produce biofilm-associated proteins (Bap_{Ab})⁵⁰, which are large surface exposed proteins orthologous to the Bap protein originally characterized in *S. aureus*⁵¹. Bap_{Ab}, which is secreted via a type I secretion system (T1SS)⁵², mediates mature *A. baumannii* biofilm formation (Fig. 1A). Specifically, Bap has a role in cell-cell adhesion and is required for the development of higher-order structures on medically relevant materials such as polystyrene and titanium⁵⁰. Although most sequenced strains of *A. baumannii* carry a *bap* gene, many seem to have disrupted or truncated *bap* sequences⁵³. It has yet to be determined if this is due to recombination events or sequence alignment errors common to the highly repetitive elements of *bap* coding sequences. Some *A. baumannii* strains also encode Bap-like proteins, BLP1 and BLP2, which coordinately contribute towards mature biofilm formation in a similar fashion as Bap_{Ab}⁵⁴. Finally, medically relevant *Acinetobacter* spp., including *A. baumannii* and *A. nosocomialis*, abundantly secrete an RTX-like domain-containing protein through the T1SS⁵². This family of proteins is orthologous to large repetitive RTX domain-containing proteins found in *Pseudomonas putida*, which mediate biofilm development⁵⁵. Other notable factors in *A. baumannii* that might be crucial for biofilm formation include the production of poly-beta-(1-6)-*N*-acetylglucosamine (PNAG)⁵⁶, which is produced by many Gram-negative species. Interestingly, antibodies against PNAG are able to eliminate *A. baumannii* in opsonophagocytosis assays, which suggests that PNAG might be a potential vaccine target⁵⁷. Other factors that contribute to biofilm formation, such as capsular polysaccharides^{58,59}, and an autotransporter system⁶⁰ are discussed below.

Motility.

In many different genera, bacterial motility is intimately linked with the ability of an organism to cause disease, for instance; in the case of *Pseudomonas aeruginosa*, the flagellum functions as a key bacterial motor that is also required for full virulence. In a related fashion, *A. baumannii* hyper-motility has been associated with enhanced virulence in a *Caenorhabditis elegans* infection model⁶¹; conversely, mutants defective in motility were shown to have an attenuated phenotype⁶². Furthermore, recent epidemiological studies of *A. baumannii* clinical isolates found blood isolates were more motile when compared against sputum isolates, indicating that motility may provide a fitness advantage in different anatomical sites⁶³.

Paradoxically, '*Acinetobacter*' translates to 'non-motile rod'; however, *A. baumannii* and *A. nosocomialis* strains are capable of two independent forms of bacterial locomotion: surface-associated motility [G] and twitching motility [G]. Twitching motility⁶⁴ is a well-described form of bacterial locomotion by many genera of bacteria. In *A. baumannii* and *A. nosocomialis*, twitching motility is dependent on fully functioning type IV pili^{65,66} for repeated rounds of extension and retraction to pull bacterial cells forward. Although a direct link between type IV pili and/or twitching motility in virulence has not been observed for *A. baumannii*, genes predicted to encode for proteins required for the biogenesis of type IV pili

were shown to be upregulated during growth in human serum⁶⁷, which indicates that type IV pili may be important during bacteremia. Moreover, almost all *A. baumannii* and *A. nosocomialis* strains carry highly homologous genes encoding for proteins required for the biogenesis of type IV pili. However, the major pilin subunit, PilA, displays remarkable sequence divergence across species and even between strains. Structural analysis of two *A. baumannii* PilA proteins and an *A. nosocomialis* PilA protein showed that these sequence divergences manifest in a high degree of structural variation, much larger than expected for a given species⁶⁸. As such, these sequence and structural variations render an *A. baumannii* type IV pilin-specific vaccine as a likely failure.

Another form of motility, termed surface-associated motility has also been observed in *A. baumannii* clinical isolates. The earliest reports of *Acinetobacter* surface-associated motility ascribed the phenomenon to be dependent on twitching motility⁶⁹. A subsequent study further strengthened these observations, as type IV pili retraction-deficient mutants in a strain of *A. nosocomialis* displayed impaired surface-associated motility⁷⁰. A third study also observed that *A. baumannii* type IV pili retraction mutants had impaired surface-associated motility⁶⁵. However, using the same *A. nosocomialis* type strain, a contradictory report found that type IV pili do not have a role in surface-associated motility⁶⁶. Specifically, it was found that *A. nosocomialis* mutants lacking functional type IV pili were unable to exhibit twitching motility, yet, displayed completely normal surface-associated motility.

A. baumannii surface-associated motility is most similar in appearance to swarming motility of *P. aeruginosa*⁷¹; but, swarming is dependent on flagella and *A. baumannii* do not produce flagella. Currently, *A. baumannii* surface-associated motility has been shown to rely on the synthesis of 1,3-diaminopropane (DAP)⁷², quorum sensing⁷⁰ and LOS production⁷³. It is possible that DAP or a derivative of DAP functions as a signaling molecule important for regulating surface-associated motility via quorum sensing. However, further work is needed to fully establish this mode of motility. Interestingly, *A. baumannii* surface-associated motility is repressed at room temperature in the presence of blue light, a feature that is dependent on proteins that contain blue light-sensing domains⁷⁴. Finally, a possible source for the conflicting results described above are the phase variable phenotypes of different *A. baumannii* and *A. nosocomialis* populations⁷⁵. Specifically, a novel phase-variable colony opacity phenotype was discovered in *A. baumannii* strain AB5075, where colonies interconvert between opaque and translucent variants. Interestingly, opaque phase variants [G], have enhanced surface-associated motility and a concomitant enhanced virulence phenotype, while translucent variants are significantly less motile⁷⁶. Thus it is possible that the previously characterized type IV pili mutants, which displayed impaired surface-associated motility phenotypes, were immotile due to a phase variable phenomenon and not an impairment with or absence of functioning type IV pili. Phase variation seems to be a common phenotype among most clinical isolates of *A. baumannii* and this phenotype is likely to control additional virulence factors. This feature must be carefully considered when designing and performing experiments assessing the virulence and fitness of pathogenic *Acinetobacter* species⁷⁶.

Interactions with hosts and competitors

Prior to the recent antibiotic resistance epidemic, *A. baumannii* was a scantily studied microorganism. In the last 10 years, many virulence factors have been uncovered as well as a thorough characterization of the innate immune response during an *A. baumannii* infection (Box 1). Virulence factors are broadly defined as molecular features used by a bacterium that enable successful interaction with and subsequent colonization of the human host. Given that nearly half of all *A. baumannii* infections are caused by MDR strains¹, there is a need to accelerate investigations into its pathobiology to find alternative ‘out-of-the-box’ strategies to combat *A. baumannii* infections⁷⁷. Two main approaches have been used to discover *A. baumannii* virulence factors. The first, unbiased approach used high-throughput transposon screenings to identify virulence factors under different experimental conditions. These approaches have identified several candidate virulence factors, mostly predictable genes involved in cellular metabolism and cell envelope biogenesis. Although powerful, this strategy has not yet led to the identification of novel toxins or pathogenesis mechanisms. This could be due to the lack of an appropriate model that recapitulates human infections, the choice of the strains or experimental conditions, or the fact that the defect in colonization by a particular mutant may be masked by the bacterial population as a whole. The second and most applied approach revolves around identifying homologous virulence mechanisms found in other human pathogens. This approach has led to the identification of protein glycosylation and secretion systems, as well as the characterization of micronutrient acquisition mechanisms, which we discuss in the following section.

Glycoconjugates

Bacterial carbohydrates, also known as glycans, provide an interface between a pathogen and its environment. Not surprisingly glycoconjugates [G] have key structural roles and mediate the first line of defense against a variety of stresses, immune evasion and regulation, and virulence in *A. baumannii* (Fig 2). Common bacterial glycoconjugates include the capsular polysaccharide, glycosylated proteins, lipopolysaccharide and peptidoglycan.

Lipopolysaccharide [G] (LPS) is a hallmark of Gram-negative bacteria and the ligand for Toll-like receptor 4 (TLR4). Similarly to *Neisseria* and *Campylobacter* species, *A. baumannii* does not contain an O antigen, thus, its LPS is appropriately designated lipooligosaccharide (LOS). Regardless, in Gram-negative bacteria, LPS or LOS is the primary component of the outer leaflet of the outer membrane and as such is generally considered an essential structural component required for bacterial viability. Moreover, the lipid anchor of LPS and LOS, termed lipid A, is the target of the cationic polypeptide antibiotic colistin, which is a last-line treatment option for carbapenem resistant *A. baumannii*⁷⁸. Not surprisingly, *A. baumannii* isolates are adapting to this antibiotic in a multitude of ways (Fig 3). Similarly to other Gram-negative bacteria, *A. baumannii* can modify its lipid A composition to deter the binding of colistin. Specifically, mutations in the *pmrAB* two-component system of *A. baumannii* mediate upregulation of *pmrA* expression⁷⁹, which is accompanied by the addition of phosphoethanolamine to lipid A and increased resistance to colistin⁸⁰. Epidemiological studies show that mutations in the *pmrAB* system are the most commonly observed mechanism for *A. baumannii* strains to become

colistin resistant^{45,81}. Other lipid A modifications implicated in colistin resistance include the addition of galactosamine⁸² and the natural presence of a predominately hepta-acylated form of LOS²⁵. Colistin resistance is also mediated by the complete loss of LOS in *A. baumannii*⁸³. This extreme adaptation, conferred by mutations in lipid A biosynthetic genes, has only been observed in *A. baumannii*, *Neisseria meningitidis* and *Moraxella catarrhalis*. Given its role in membrane integrity and stability, loss of lipid A, and thereby LOS, in *A. baumannii* requires compensatory mechanisms to support viability, such as the overexpression of certain lipoproteins as a form of outer membrane stabilization⁸⁴. Another noted change in LOS-deficient strains includes an increase in the capsular polysaccharide poly-beta-1,6-*N*-acetylglucosamine⁸⁵; however, this compensatory mechanism has not been universally observed⁸⁴.

Most *A. baumannii* strains also carry a thick capsular polysaccharide⁵⁸ and an *O*-linked protein glycosylation system⁵⁹. Capsular polysaccharides protect bacteria from external threats, including host defenses. Protein glycosylation is the process of transferring a carbohydrate, usually an oligosaccharide, to a protein. In *A. baumannii* and *A. nosocomialis*, both the capsular polysaccharide and the *O*-glycans that decorate proteins share a biosynthetic pathway^{86,87}. These processes start with the assembly of a lipid-linked oligosaccharide at the cytoplasmic side of the inner membrane. Once assembled, the lipid-linked oligosaccharide is flipped to the periplasmic side of the inner membrane. Upon reaching the periplasmic side, the lipid-linked oligosaccharide can be directly transferred to a protein by an oligosaccharyltransferase or further processed by the Wzy polymerase into repeating polysaccharide units required for capsule biogenesis (Fig. 2).

The capsular polysaccharide is mainly responsible for the extraordinary resistance to complement-mediated killing [G] exhibited by most *A. baumannii* strains^{58,86}. In fact, the capsular polysaccharide of *A. baumannii* could be considered its main virulence factor as strains lacking the capsule are avirulent and readily killed by complement⁸⁶. The glycans attached to proteins, which are most often located in the periplasm or the outer membrane and are exclusively membrane associated in *A. baumannii*, are not involved in resistance to complement, but cells lacking the glycosylation system are defective in biofilm formation and are attenuated in several infection models, which indicates a role for protein glycosylation in the successful adaptation to the host environment⁵⁹. Moreover, most *A. baumannii* and *A. nosocomialis* strains contain two *O*-glycosylation [G] systems: one system is responsible for glycosylation of multiple proteins of unknown function and the other system has been implicated in the decoration of type IV pilin with glycans⁸⁷. The biological role of protein glycosylation remains elusive; however, recent structural analyses and glycan modeling indicated that the pilin glycans may have evolved to shield the protein components from antigenic recognition⁶⁸.

Given the importance of capsular polysaccharides to *A. baumannii* virulence and the successful implementation of many capsular polysaccharide-conjugate based vaccines for the prevention of *Streptococcus pneumoniae*, *Haemophilus influenzae* type B and *N. meningitidis*, an *A. baumannii* capsule-based conjugate initially seemed promising. Unfortunately, the variability among glycan structures, including capsule and glycoproteins, and the glycan core, is outstanding, and often includes sugars that have not previously been

identified in any other species²³. Clearly, this will make the formulation of an effective glycan-based vaccine difficult, if not impossible. Adding to the complexity, capsule synthesis can be increased upon contact with sub-inhibitory concentrations of antibiotics, in a mechanism mediated by the two-component regulatory system BfmRS, which suggests that improper antibiotic therapy may further enhance the virulence of *A. baumannii*⁸⁸. Nevertheless, inhibitors of the glycan synthesis pathway would be highly valuable therapeutics to combat MDR *A. baumannii* infections.

Micronutrient acquisition systems.

Infecting the human host requires a coordinated response from *A. baumannii* that not only impairs cellular defense mechanisms, mainly in the form of protection via the capsular polysaccharide, but also enables for metabolic and nutritional flexibility. Transition metals such as iron, manganese and zinc are essential for all domains of life; as such, hosts have evolved elaborate mechanisms to sequester metals, a process that is referred to as nutritional immunity. One key to the success of *A. baumannii* as a nosocomial pathogen is its diverse mechanisms for scavenging these scant nutrients *in vivo*. This is particularly evident in the form of high-throughput transposon screens, where mutants that contain transposon insertions within iron and zinc import and/or utilization genes are severely attenuated in virulence models^{89–91}.

The primary mechanism used by *A. baumannii* for scavenging iron is mediated through the action of high affinity iron chelating molecules known as siderophores [G]. The most commonly conserved iron chelating agent in *A. baumannii* is the catechol-hydroxymate siderophore, acinetobactin⁹². Depending on the pH of the extracellular environment, acinetobactin can isomerize into one of two forms, an oxazoline- or isooxazolidinone-containing form, both of which chelate iron⁹³. This unique isomerization feature enables acinetobactin to bind iron under acidic conditions found during acute infections. Importantly, acinetobactin is absolutely required for virulence⁹⁴, thus rendering its synthetic pathway an attractive antibacterial target. Another other sets of catechol-hydroxymate siderophores, fimsbactin A-F⁹⁵ as well as the hydroxymate siderophores baumannoferrin A and baumannoferrin B⁹⁶, are also iron scavengers used by *A. baumannii*; however, a detailed genetic and molecular analysis of their biosynthetic and transport machinery is lacking.

Zinc, which is a structural cofactor for many proteins, is also essential for the survival of *A. baumannii*. Given its essentiality to many bacterial pathogens, mammalian systems have evolved zinc sequestration mechanisms, including the production of the zinc chelating protein calprotectin⁹⁷. Calprotectin production and release is robustly induced upon *A. baumannii* infection in the lungs of mice and persists for the entirety of the infection⁹⁸. To combat calprotectin-mediated nutritional immunity, *A. baumannii* relies on a high affinity zinc acquisition system, termed ZnuABC⁹⁹. The ZnuABC system is regulated by the zinc uptake regulator (Zur), which functions as a transcriptional repressor that binds to conserved DNA motifs upstream of many zinc-regulated genes, thus blocking their expression¹⁰⁰. Under zinc-depleted conditions or in the presence of calprotectin, intracellular zinc levels decrease and Zur-mediated repression is relieved. A second Zn-uptake system, also regulated by Zur, relies on the coordination between the metallochaperone, ZigA, and the

histidine utilization (Hut) system for the uptake and release of histidine bound zinc (His-Zn) complexes¹⁰¹. In this system, the His-Zn complexes are imported by the outer membrane transporter HutT. Once inside *A. baumannii* cells, zinc is liberated from the His-Zn complexes to a bioavailable form with the coordination of ZigA and the histidine ammonia lyase, HutH. Like siderophores, zinc utilization systems are attractive antimicrobial targets given their importance *in vivo* and the lack of homologous systems in eukaryotic organisms.

Recently, the importance of micronutrient acquisition and metabolism were linked in *A. baumannii* virulence. Specifically, *A. baumannii* subverts host-mediated metal limitation through the concerted action of a manganese import system and subsequent urea metabolism¹⁰². It is unclear why urea metabolism is important for *A. baumannii* growth under metal-limited conditions; however, it was speculated that metal limitation may cause a metabolic build-up of urea that requires manganese-mediated breakdown. Clearly, these types of studies show the importance of micronutrient acquisition systems towards the virulence potential of *A. baumannii* and further emphasize the potential of those systems as novel antimicrobial targets.

Protein secretion.

Similar to other Gram-negative pathogens, *A. baumannii* uses secreted protein products to facilitate environmental and host adaptation. Within the last five years, type I⁵², type II^{103,104}, type IV¹⁰⁵, type V⁶⁰ and type VI^{106,107} secretion systems have been uncovered in *A. baumannii* (Fig. 1). For a detailed review on *Acinetobacter* secretion systems and secreted proteins, the reader is referred to the following review¹⁰⁸.

The first secretion system identified in *A. baumannii* was the *Acinetobacter* trimeric autotransporter (Ata)⁶⁰, a type Vc autotransporter, which consists of a membrane-associated transporter domain and a large, repetitive passenger domain that extrudes through the transporter domain (Fig. 1A). Ata is important for adhesion [G] to host extracellular matrices and basal membrane components⁶⁰. Ata is present in many clinical isolates and may be a potential vaccine candidate. Indeed, passive administration of anti-Ata serum protects neutropenic mice from infection¹⁰⁹. However, Ata expression is variable amongst clinical isolates and thus may not provide adequate coverage for a comprehensive vaccine⁶⁰.

A. baumannii and *A. nosocomialis* strains also use a type VI secretion (T6SS) system for bacterial competition^{106,107} (Fig. 1C). This is particularly important in the context of polymicrobial infections as the *Acinetobacter* T6SS has not been found to mediate eukaryotic cytotoxicity; yet, it provides an *in vivo* fitness advantage given its potent anti-bacterial activity. T6SS are often tightly regulated through various control mechanisms. This holds true for *A. baumannii*, as expression of the T6SS components and activity can vary greatly between strains. Whereas some strains are constitutively expressing their T6SS and release effectors *in vitro*, some strains have developed exquisite forms of regulation. For example, several *A. baumannii* strains contain a large conjugative plasmid that harbors many antibiotic resistance genes as well as repressors of the T6SS¹¹⁰. Specifically, two TetR-like regulators encoded on the plasmid repress T6SS activity, which is chromosomally encoded. Upon spontaneous loss of the plasmid, cells are relieved of T6SS repression and become potent bacterial killers. At the same time, cells that lost the plasmid now become susceptible

to antibiotics, given that many resistance cassettes are located on the plasmid. This novel form of regulation may be altruistic as cells that lose the plasmid may be able to defend the entire population against competing bacteria in the polymicrobial environment. When antibiotics are introduced, cells that contain the plasmid survive and thus ensure the survival of the population.

A. baumannii and *A. nosocomialis* also use a type II secretion system (T2SS) for the export of multiple effector proteins^{103,104} (Fig 1B). Two of these effectors, the lipase LipA and the metalloprotease CpaA, require dedicated chaperones, LipB and CpaB respectively, underscoring an under recognized area of T2SS dynamics¹⁰⁴. Previously, it was thought that the type III secretion system was the only secretion system that specifically used widespread chaperones for secreted effectors. Importantly, the *A. baumannii* and *A. nosocomialis* T2SSs functions as a *bona fide* virulence factor secreting effectors that mediate colonization of the lung and dissemination to other organs. Moreover, it was recently shown that CpaA may be one of the main virulence factors secreted by the T2SS as a *cpaA* mutant was less virulent in both an invertebrate model and a murine model of pneumonia¹¹¹. Interestingly, the *cpaA* mutant was less able to disseminate to the spleen. This may be due to the ability of CpaA to decrease blood coagulation¹¹², a process that may help *A. baumannii* disseminate throughout the bloodstream to other anatomical sites, like the spleen. However, the role of each specific effector, other than CpaA, in *A. baumannii* and *A. nosocomialis* pathogenesis remains elusive. Collectively, future studies are needed to define the secreted proteins that contribute to the success of *A. baumannii* as a nosocomial pathogen.

Conclusions and future perspectives

There are well over 2,000 *A. baumannii* genome sequences publicly available. Impressively, the paralog-collapsed pan-genome size of *A. baumannii* reaches almost 12,000 sets of genes¹¹³. Furthermore, these types of analyses enable the mapping of the core *A. baumannii* genome. From this, the global distribution across *A. baumannii* strains and even *A. nosocomialis* strains of essential virulence factors, like the capsular polysaccharide, secretion systems, and micronutrient acquisitions systems are readily identifiable. Together with the accumulation of resistance mechanisms, the success of *A. baumannii* as a nosocomial pathogen becomes evident. However, what if *A. baumannii* does acquire a toxin-like virulence factor similar to other human pathogens, such as, *Vibrio cholerae* or *Clostridium difficile*? Molecular studies have shown that *A. baumannii* has many of the same secretion systems as other well-known pathogens and although there is no evidence to indicate such an acquisition, the possibility does exist.

In parallel, the emergence of multidrug resistant *A. baumannii* emphasizes the urgency to develop alternative treatment strategies. These anti-virulence strategies may manifest in the form of phage-therapy, metabolic interference therapy, antimicrobial peptide therapy or vaccine strategies⁷⁷. One such example would be targeting the phenylacetic acid (PAA) catabolic pathway with selective inhibitors as PAA functions as a potent chemoattractant for neutrophils¹¹⁴. Recently, an *A. baumannii* mutant auxotrophic for D-glutamate was shown to be a very promising live attenuated vaccine candidate given its inability to synthesize mature peptidoglycan¹¹⁵.

Finally, it is clear that recent clinical isolates are genetically distinct from type strains commonly used for scientific studies⁴⁵. The two most used strains, *A. baumannii* ATCC 17978 and *A. baumannii* ATCC 19606, have been excellent models for over two decades; however, both strains were isolated almost 70 years ago and do not adequately reflect clinical isolates that were recovered in the past three decades. As an example, *A. baumannii* ATCC 17978 carries the pAB3 plasmid, a large conjugative plasmid containing a single antibiotic resistance cassette. Over the course of 60 years, this plasmid has evolved into the pAB04 plasmid which now contains 12 resistance cassettes in the same locus, which indicates that antibiotic resistance has been positively selected. Another example is CpaA, which is commonly found in recent clinical isolates but is absent in both ATCC 17978 and ATCC 19606. Therefore, the use of modern clinical isolates, such as *A. baumannii* AB5075¹¹⁶, will provide a more contemporary understanding of the molecular mechanisms important for survival and adaptation in this era and may lead to the identification of more relevant virulence factors outside of those involved in the persist and resist strategy commonly recognized as the only virulence mechanism of *A. baumannii*.

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GLOSSARY

Capsule

an extracellular polysaccharide layer that encompasses the entire bacterium, which acts as a glycan shield protecting the bacterium from many external threats

Lipooligosaccharide (LOS)

a macromolecule consisting of lipid-A and a core oligosaccharide found in the outer leaflet of the outer membrane of Gram-negative bacteria. Lipid-A is also considered endotoxin and is the ligand for toll-like receptor 4

Phagocytosis

the process used by many immune cells, including macrophages, to engulf invading bacteria

Catalase

an enzyme that detoxifies hydrogen peroxide into water and oxygen

Two-component regulatory system

a two part relay system employed by bacteria used for sensing and responding to environmental stimuli, consisting of a membrane bound histidine kinase and a soluble response regulator

Surface-associated motility

a mechanism of bacterial translocation observed on semi-solid surfaces unique to *Acinetobacter* spp., which is not dependent on pili

Twitching motility

a mechanism of bacterial translocation dependent on repetitive rounds of type IV pili extension and retraction broadly used by many bacteria

Opaque phase variants

a subset of an *A. baumannii* population that has an opaque appearance when viewed under a dissecting microscope, which varies from the translucent form in terms of both appearance and virulence

Glycoconjugates

macromolecules composed of a carbohydrate covalently attached to at least one other lipid or protein molecule

Lipopolysaccharide (LPS)

a macromolecule consisting of lipid-A, a core oligosaccharide, and a polysaccharide O-antigen found in the outer leaflet of the outer membrane of Gram-negative bacteria

Complement-mediated killing

part of the innate immune system consisting of soluble proteins in the blood that coordinately binds to an invading pathogen, triggering either lysis or the recruitment of immune cells to clear the pathogen

O-glycosylation

the covalent attachment of a carbohydrate moiety to the hydroxyl group of a serine or threonine in a polypeptide

Siderophores

high affinity iron binding molecules secreted by many bacterial pathogens to scavenge for iron

Adhesion

the process of a bacteria associating with a surface, either biotic like human cells or abiotic in the form of medical equipment and devices

Author Biographies

Christian M. Harding received a Ph.D. in Biomedical Sciences from The Ohio State University. For over seven years, he has studied the molecular mechanisms of *Acinetobacter* pathogenesis, including *Acinetobacter* surface appendages, secretion systems, and glycoconjugates. He is now Co-Founder and Chief Scientific Officer of VaxNewMo LLC, a biotech startup dedicated to making conjugate vaccines using a glycoengineering approach in the lab safe *Escherichia coli*.

Seth W. Hennon earned a Ph.D. in Biochemistry at The Ohio State University studying membrane protein biogenesis. He is currently a postdoctoral researcher in the laboratory of Mario Feldman at the Washington University in St. Louis, where his research focuses on the biogenesis of the Type VI secretion system in *Acinetobacter*.

Mario F. Feldman obtained his Ph.D. at the University of Buenos Aires, Argentina. During his postdoctoral training, he focused on the study of type 3 secretion systems and on bacterial protein glycosylation. He started as independent researcher in 2006 at the University of Alberta, Canada, then moved to Washington University in St Louis in 2015. His group investigates the glycobiology and pathogenesis of *Acinetobacter*, and the biogenesis of outer membrane vesicles. He is the founder of VaxAlta Inc. and VaxNewMo LLC., companies focused on antibacterial vaccines.

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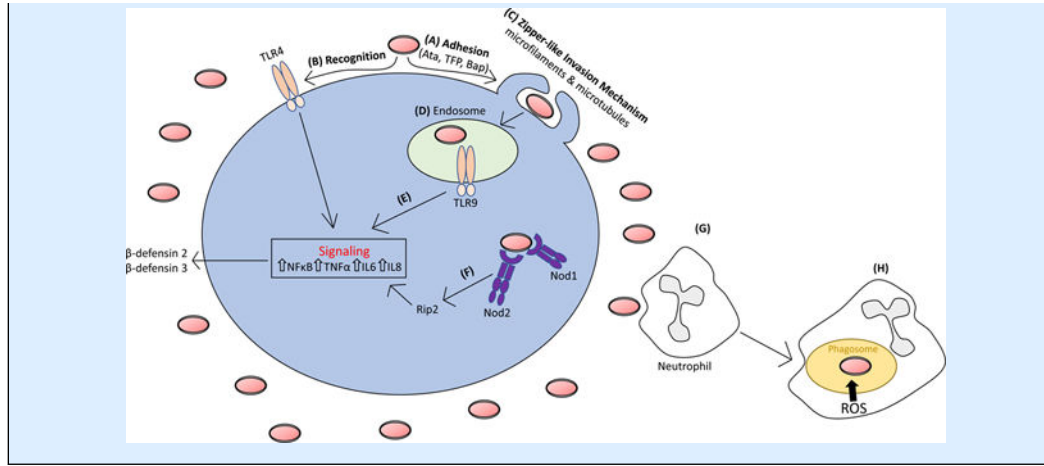
KEY POINTS

- *Acinetobacter baumannii* is an opportunistic human pathogen that predominantly causes healthcare-associated infections.
- Many members from the genus *Acinetobacter*, including, *Acinetobacter nosocomialis*, *Acinetobacter pittii*, *Acinetobacter dijkschoorniae* and *Acinetobacter seifertii*, are also human pathogens and increasingly identified as the cause of infections.
- *A. baumannii* is rapidly developing resistance mechanism to antibiotics.
- The ability of *A. baumannii* to withstand desiccation and to form biofilms promotes its success as a nosocomial pathogen.
- Fundamental virulence factors, such as surface adhesins, glycoconjugates and secretion systems directly contribute to the pathogenesis of *A. baumannii*.

Innate immune response to *Acinetobacter baumannii* infection

As with many pathogenic microorganisms, *A. baumannii* interacts with mucosal epithelial cells¹¹⁷ as well as endothelial cells in the case of bloodstream infections¹¹⁸. These interactions, mediated through adhesins, like Ata¹¹⁸, type IV pili⁶⁸, and Bap¹¹⁹, provide *A. baumannii* intimate contact with host substratum (see the figure). At the cell surface, key innate immune components known as pattern recognition receptors (PRRs) can then recognize specific pathogen associated molecular patterns (PAMPs) of *A. baumannii*. The extracellular PRR Toll-like receptor 4 (TLR4), which recognizes lipid A of LOS, has a prominent signaling role helping to limit *A. baumannii* burden in both pneumonic and systemic infection models. Although *A. baumannii* is considered an extracellular pathogen, it can invade epithelial cells using a zipper-like mechanism for uptake, which is dependent on both microfilaments and microtubules¹²⁰. *A. baumannii* can persist within membrane bound vacuoles in the cytoplasm necessitating intracellular PRRs for pathogen detection. Indeed, signaling from the endolysosomal PRR, TLR9, which detects bacterial DNA, limits *A. baumannii* pneumonia and bacterial dissemination¹²¹. Furthermore, the cytosolic PRRs NOD1 and NOD2 as well as their downstream signaling partner RIP2 limit intracellular *A. baumannii* proliferation in epithelial cells, which indicates that *A. baumannii* can escape membrane-bound vacuoles; however, their escape mechanisms are not yet known¹²². Ultimately, recognition by PRRs results in downstream signaling cascades mediated by the activation of NF- κ B, the mitogen-activated protein kinase pathway, and concomitant pro-inflammatory cytokine production that include TNF- α , IL-6 and IL-8^{122,123}.

Upon detection, epithelial cells secrete antimicrobial peptides; such as; human β -defensin2 and human β -defensin 3, which functions as a first line of defense, inhibiting the growth of invading *A. baumannii*^{123,124}. Simultaneously, recruitment of phagocytic cells to the site of *A. baumannii* infection is mediated by cytokine and chemokine signaling. Neutrophils are the first to arrive and most important phagocytic cell for controlling *A. baumannii* infections¹¹⁴. This is particularly evidenced through antibody-mediated neutrophil depletion^{125,126}, which greatly enhances *A. baumannii* pathogenesis resulting in severe mortality in experimental models. Mechanistically, neutrophil clearance of *A. baumannii* is mediated by the activity of the NADPH oxidase system, which generates reactive oxygen species to kill phagocytosed bacteria¹²⁷, with limited roles for reactive nitric species. Other phagocytic cells like macrophages and dendritic cells play minor roles in *A. baumannii* clearance, but may play important sensing and signaling roles.

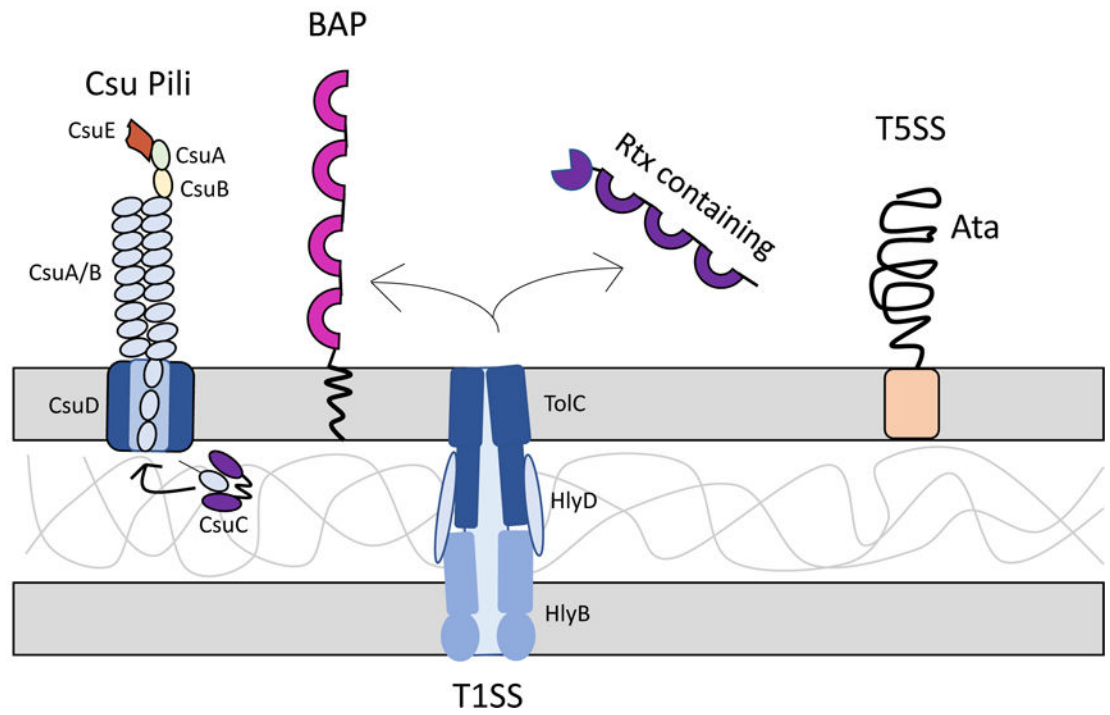


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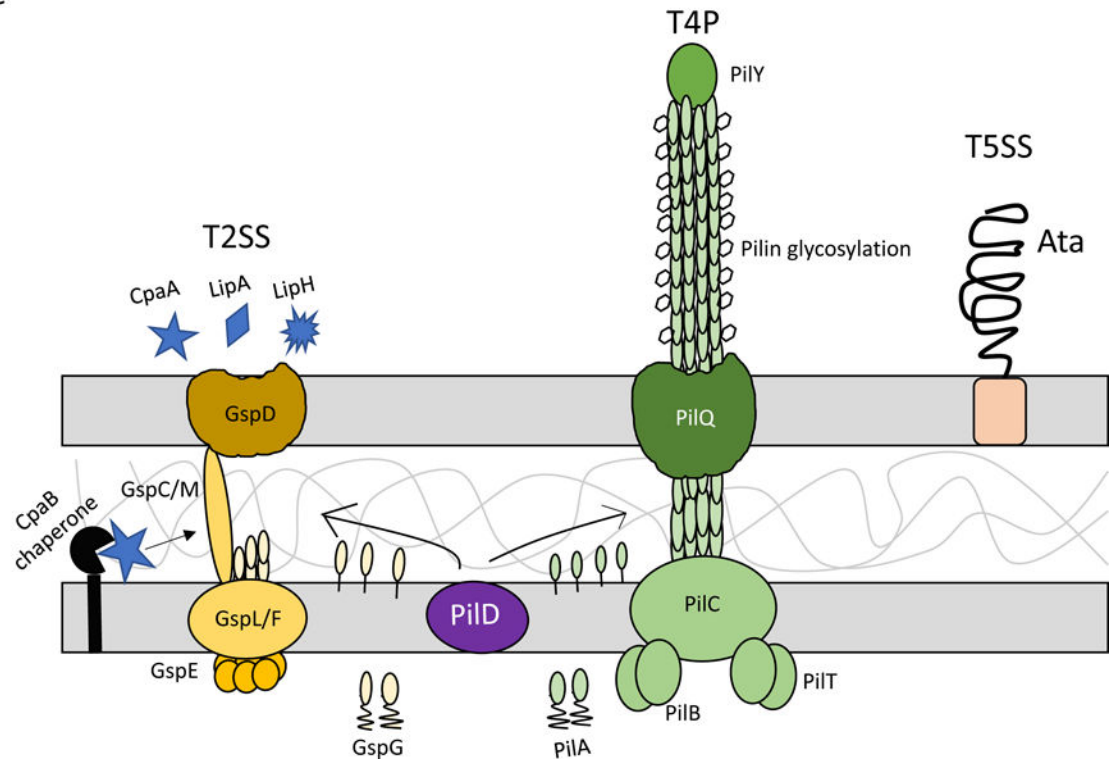
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(A) Biofilm Formation

(B) Bona fide human virulence factors



(C) Bacterial Competition (Contact Dependent)

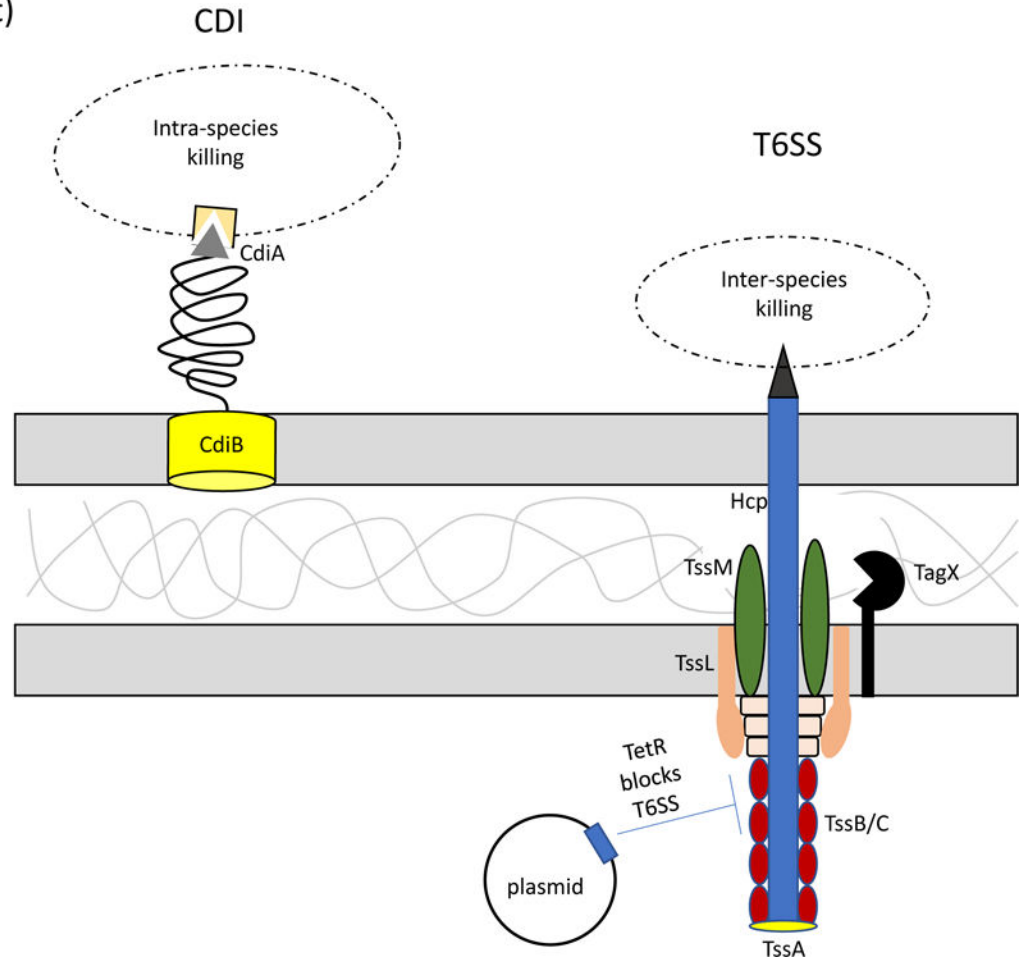
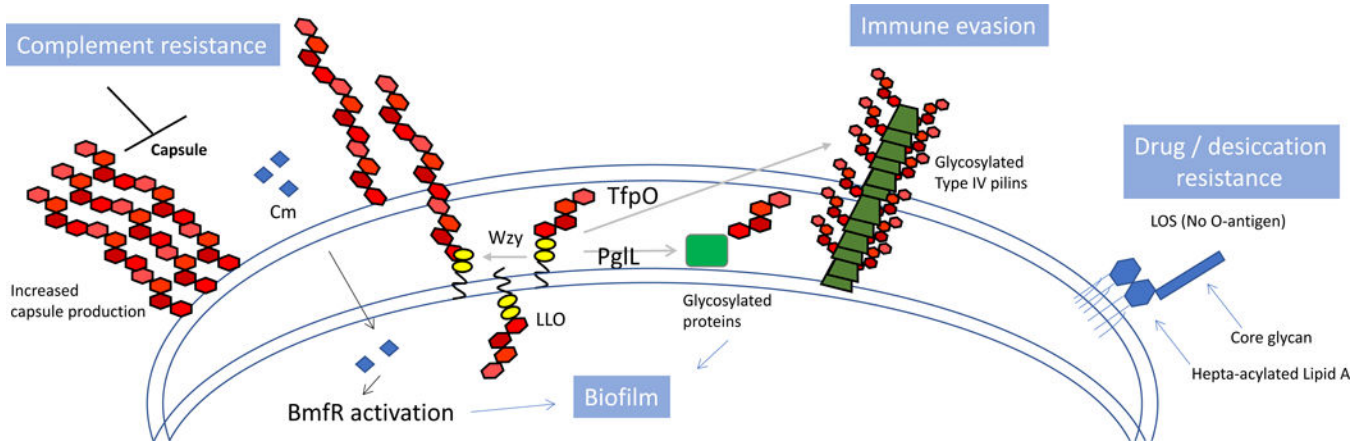


Figure 1: Protein secretion and export in *Acinetobacter baumannii*.

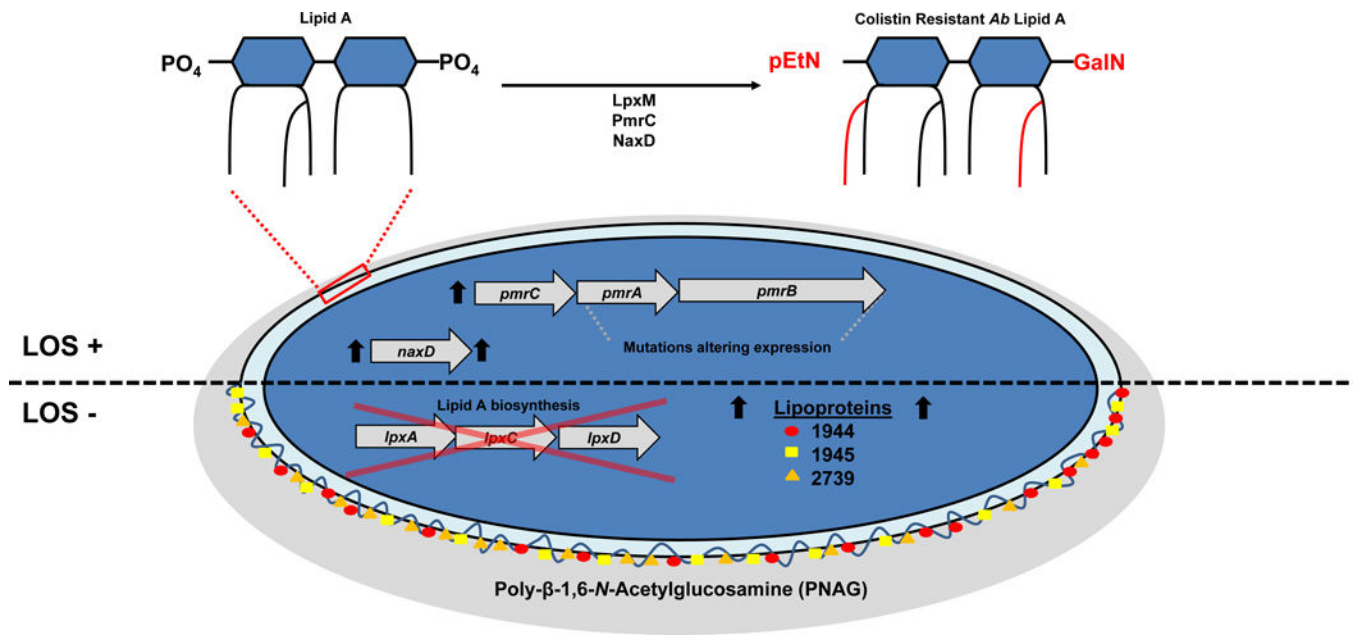
Secretion systems and extracellular appendages of *Acinetobacter baumannii* are involved in the formation of biofilms, in virulence and bacterial competition. **(A)** The Csu system extrudes a type I chaperone-usher pilus that is essential for the formation and maintenance of biofilms on abiotic surfaces, which may contribute to persistence in the hospital environments⁴³. The CsuA/B pilin subunits are trafficked to the outer membrane CsuD usher by CsuC chaperones. The CsuD usher facilitates polymerization of CsuA/B monomers into a pilin fiber. Pilin polymerization is initiated by the CsuE tip adhesion and the minor pilins CsuA and CsuB, all of which are also trafficked to the CsuD usher by CsuC chaperones. Biofilm-associated proteins (Bap)⁵⁰ and an effector protein that contains an RTX-like domain are secreted by the type I secretion system (T1SS)⁵² and are involved in the formation and stability of mature biofilms. The T1SS is composed of the outer membrane protein TolC, the periplasmic adaptor protein HlyD, and the inner membrane ATPase HlyB. The *Acinetobacter* trimeric autotransporter (Ata) type Vc secretion system consists of a membrane-associated transporter domain and a large, repetitive passenger domain that extrudes through the transporter domain⁶⁰. The adhesin promotes adherence to extracellular and basal membrane components of the host. **(B)** The type II secretion system (T2SS)

secretes multiple effectors that were shown to be required for virulence *in vivo*, including the lipases LipA and LipH as well as the protease CpaA^{103,104}. Novel, dedicated chaperones are required for two of the effectors, including the CpaB chaperone, which is required for the secretion of CpaA, the most abundant type II effector. *A. baumannii* and *A. nosocomialis* also produce type IV pili, surface appendages evolutionarily related to the T2SS⁶⁶. In *A. baumannii* and *A. nosocomialis* these two systems share a processing protein, PilD, required to process pre-pseudopilins and pre-pilins prior to assembly into the T2SS and type IV pilus, respectively¹⁰⁴. Type IV pili are also glycosylated in *A. baumannii* and *A. nosocomialis* as a possible form of antigenic variation to evade host detection. Specifically, the pilin glycan is predicted to mask the major pilin subunit, PilA, from antibody recognition; furthermore, PilA displays remarkable sequence divergence across species and even between strains leading to reduced immune recognition in the case pilin subunits are not glycosylated⁶⁸. (C) Contact-dependent secretion systems are used by *A. baumannii* for inter- and intra-species killing to eliminate bacterial competitors. Many strains of *Acinetobacter* have two distinct contact-dependent inhibition (CDI) systems to kill sister cells that do not have the associated immunity protein⁵². The type VI secretion system (T6SS)^{106,107} is used for inter-species competition and contains a novel *L,D*-endopeptidase, TagX, which is required for transit of the machinery through the peptidoglycan layer¹²⁸. A large, conjugative plasmid which also contains drug-resistance genes (not indicated) regulates the expression of T6SS in some clinical isolates¹¹⁰. The individual components of the secretion systems are indicated in all panels.



Acinetobacter baumannii surface-exposed glycoconjugates.

The capsular polysaccharide, glycoproteins and hepta-acylated lipooligosaccharide (LOS) all contribute to virulence of *Acinetobacter baumannii*. In *A. baumannii* and *A. nosocomialis*, the capsular polysaccharide and glycoproteins are formed by glycans alone or glycans attached to proteins, respectively⁵⁹. Glycan synthesis is initiated at the inner membrane by dedicated glycosyltransferases that transfer sugars to a phosphorylated lipid generating a lipid-linked oligosaccharide (LLO). The LLO is then flipped to the periplasm where the glycan component can be transferred by PglL, an oligosaccharyltransferase (OTase) to proteins in the periplasm or outer membrane, or to type IV pilins by TfpO, a pilin specific OTase. The LLO can also be further processed and polymerized into a repeating polysaccharide by the Wzy polymerase prior to transport to the outer membrane for the capsule. In *A. baumannii* the capsular polysaccharide protects cells from complement-mediated killing⁵⁸. *A. baumannii* glycoproteins contribute to virulence by enhancing biofilm formation and maintenance⁵⁹, while glycosylated type IV pilins⁸⁷ have been implied to function in immune evasion, shielding the antigenic protein from antibody recognition⁶⁸. Finally, the hepta-acylated LOS, consisting of a core glycan and lipid A, lacks an O-antigen but directly contributes to drug and desiccation resistance²⁵.



Colistin resistance mechanisms of *A. baumannii*.

In *Acinetobacter baumannii*, colistin resistance manifests either through modifications of the lipid anchor of LOS, termed lipid A, or by the complete loss of LOS, both of which alter binding affinity of colistin. Most commonly, colistin-resistant clinical isolates harbor mutations in the two-component regulatory system PmrA-PmrB, which is associated with the addition of phosphoethanolamine (pEtN) by PmrC¹²⁹, and galactosamine (GalN)⁸² to lipid A that presumably alter the binding affinity of colistin. The addition of GalN is dependent on the deacetylase activity of NaxD¹³⁰, which is regulated by the PmrA-PmrB system. Moreover, the dual activity of the acyltransferase, LpxM, leads to the constitutive expression of a predominately hepta-acylated form of LOS, which provides increased resistance against colistin²⁵. In extreme cases, *A. baumannii* will acquire mutations in the lipid A biosynthetic pathway thereby halting its production⁸³. In the absence of lipid A, *A. baumannii* has been shown to upregulate lipoproteins, namely A1S_1944, A1S_1945 and A1S_2739, which stabilize the outer membrane⁸⁴. The accumulation of the capsular polysaccharide poly-β-1,6-*N*-Acetylglucosamine (PNAG) has also been observed in strains lacking lipid A as a proposed mechanism of membrane stabilization⁸⁵.