The Type II Secretion System in *Acinetobacter baumannii*:

Its Role in Pathogenesis and Translational Implications

By

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Dedication

For: Eric, Rose, Mom, Dad, Hannah, Louis, and Emma.

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Contents

List of Tables

List of Figures

Abstract

Acinetobacter baumannii is a Gram-negative, nosocomial pathogen that typically infects immune-compromised individuals. It is of growing concern in healthcare settings due to its ability to form biofilms, survive long periods of desiccation, and easily acquire antibiotic resistance. In an effort to better combat this pathogen, we have focused on elucidating aspects of the pathogenesis of *A. baumannii*. Many Gram-negative bacteria such as *Vibrio cholerae* and *Pseudomonas aeruginosa* possess a Type II Secretion System (T2SS) to secrete substrates used for colonization and survival in their environment. In our first study, we highlighted the unique organization of the T2SS genes, *gsp*, in *A. baumannii* and demonstrated that, despite this uniqueness, the T2SS is functional by analyzing a secreted lipase, LipA. Unlike the wild-type ATCC 17978 strain, both the Δ*lipA* and Δ*gspD* mutant strains were unable to grow on media where long-chain fatty acids were the only source of nutrients. This difference was also observed in a quantitative lipase assay using 4-nitrophenyl myristate as the substrate for LipA. To determine if the T2SS has a role *in vivo* we inoculated mice via tail vein with an equal number of the wild-type 17978 strain and a mutant strain (either the Δ*lipA* or Δ*gspD* strain). Both mutants were outcompeted by the wild-type strain indicating that the T2SS is necessary for full colonization in a bacteremia model.

We continued exploring the role of the T2SSs in pathogenesis by characterizing the metalloprotease CpaA secreted by the *A. baumannii* strain AB031. We verified that CpaA is a T2S substrate using an Activated Partial Thromboplastin Time (aPTT) assay, which measures the function of the intrinsic coagulation pathway, the target of CpaA. A modified aPTT assay demonstrated that CpaA targets the coagulation component factor XII (fXII). We pinpointed the site of cleavage between Pro308 and Thr309 by subjecting cleaved fXII to N-terminal sequencing by Edman degradation. As Thr309 is O-glycosylated, we tested for the necessity of O-glycosylated residues by treating fXII with deglycosylases. CpaA was unable to cleave deglycosylated fXII, but deglycosylation did not affect the function of fXII when measured by an aPTT assay. Finally, we utilized a murine bacteremia model and observed that the strain lacking CpaA was unable to compete with the wild-type strain, suggesting that it contributes to *in vivo* fitness possibly by preventing intravascular capture by the coagulation system.

In addition to the virulence traits mentioned above, the T2SS also aids in survival in serum as the wild-type strain, 17978, withstands complement mediated killing via the alternative complement pathway significantly better than a T2SS mutant. As the T2SS contributes to *in vivo* survival and protection from human complement, we designed, optimized, and validated a high-throughput screen (HTS) to identify inhibitors of the T2SS in *A. baumannii*. By targeting the T2SS a putative inhibitor would block the secreted substrates and, thereby, their functions. By utilizing a whole-cell lipase assay, we developed a HTS with low variation among the controls and a dynamic range between the positive and negative controls, giving a z-factor of 0.65. In addition, we showed that our screen is robust, sensitive, and reproducible. Taken together, my thesis

work provides much needed insight into the pathogenesis of *A. baumannii* by elucidating the role of the T2SS and its substrates, LipA and CpaA.

Chapter I:

Introduction

Acinetobacter baumannii Infection

Acinetobacter baumannii is a Gram-negative, global nosocomial pathogen that has developed multi-drug resistance (1-3). Several risk factors increase the chance of developing an *A. baumannii* infection, including but not limited to admission to the ICU, having invasive devices or extensive wounds, or having recently taken antibiotics (4-6). While *A. baumannii* has a reputation as a nosocomial pathogen that only targets the immune-compromised, this may no longer be the case. Recently isolated, highly virulent strains have caused fatal infections in patients with relatively low co-morbidity, and illness scores and these strains are capable of killing mice without compromising the immune system prior to infection (7, 8) . *A. baumannii* causes pneumonia, bacteremia, meningitis, urinary tract infection, and skin and wound infection that may lead to sepsis (6, 9). In addition to causing clinical symptoms, *A. baumannii* can survive long periods of desiccation, and this persistence can lead to easy transmission of the disease from contaminated tubing, bed rails, bedding, and sinks (10). Understanding how *A. baumannii* can cause infection and survive in its environment is vital to combating this disease.

Antibiotic Resistance

A. baumannii is one of the ESKAPE pathogens, along with the pathogens *Enterococcus faecalis*, methicillin-resistant *Staphlyococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterbacter* species (11). These pathogens are concerning not only because they cause the bulk of nosocomial infections in the United States, but also because they are either highly pathogenic, highly antibiotic-resistant, or both (11). Antibiotic resistant strains of *A. baumannii* have surged, due in part to its ability to acquire DNA through natural transformation (12). When nosocomial infections of *A. baumannii* were first identified, they were easily treated with antibiotics. Recently, many multi-drug resistant strains, which are resistant to at least three classes of antibiotics, have been isolated as well as pan-resistant strains which are resistant to at least one member of all known antibiotic classes (6, 13).

At sub-minimal inhibitory concentrations (MIC), antibiotics are unable to stop a bacterial infection. Moreover, antibiotic use results in an increase in bacterial exopolysaccharide production and a concomitant increase in resistance to some classes of antibiotics (14). In addition, increased capsule production also confers resistance to complement-mediated killing in serum (14). This two-pronged effect is especially troubling given the dual problem of over-prescription of antibiotics and the lack of compliance with taking a full antibiotic regimen.

To prevent antibiotics from even reaching their target, *A. baumannii* has ways to remove the antibiotics or block their entry. Efflux pumps can actively remove antibiotics from the cells. Resistant nodulation cell division efflux pumps are upregulated in many

multi-drug resistant strains of *A. baumannii.* Overexpression of these pumps does come with a small fitness cost, most likely due to the increase of energy needed, but also increases virulence (15). Acylation of lipid A of *A. baumannii* lipopolysaccharide by the LpxM-dependent acylation system is important for resistance to vertebrate cationic antimicrobial peptides (CAMPs) as well as to polymyxins B and E (16) as it prevents the antibiotics from even reaching the cell membrane. These are just two of the possible methods for preventing antibiotic access.

When antibiotics are administered in high enough dosage to enter and damage the bacterium, *A. baumannii* has additional systems to protect itself. The protein RecA, which repairs DNA damage, plays a protective role against some antibiotics. For example, a *recA* mutant of *A. baumannii* is more sensitive to β-lactams, colistin, trimethoprim-sulfamethoxazole, and quinolone-type antibiotics (17).

In some cases, the acquisition of colistin resistance can lead to a loss of virulence, such as when there is a mutation in the *lpxACD* genes (18). However, a mutation in a different gene, *pmrB*, increases colistin resistance without a loss of virulence (18). Like many bacteria, *A. baumannii* develops and uses a wide variety of mechanisms to survive antibiotic treatment. The mechanisms by which *A. baumannii* is killed by antimicrobials are diverse, and work continues in this area to guide further treatment options as well as to limit the occurrence of resistant strains.

Mechanisms of Pathogenesis

The symptoms of some bacterial infections can sometimes be attributed to one or a few toxins or virulence factors. Examples of this include the cholera toxin produced by *Vibrio cholerae* or toxin A and toxin B from *Clostridium difficile*. However, this is not the case with *A. baumannii*. Despite the growing body of work on *A. baumannii* pathogenesis, no one factor nor even a small subset of factors has been found to be solely responsible for the clinical outcomes of the disease. However, strains with increased virulence have been isolated recently, and, while they need further investigation, it is possible that they may have acquired new gene(s) that contribute to virulence.

One of the main contributions to pathogenesis of *A. baumannii* is the bacterium's ability to persist on surfaces and survive desiccation for long periods. The RecA protein mentioned earlier (which aids in DNA repair) increases bacterial survival in dry conditions, as it repairs the damage caused by desiccation (17). Acylation of lipid A is also implicated in protection from desiccation, as loss of fatty acids in the outer membrane due to a lack of the glycotransferase LpxM leads to a decrease in survival in dry conditions (16). Biofilms also increase *A. baumannii*'s persistence on surfaces (19), and in fact, may be one of the main contributions to its survival. The sensor kinases BfmR (20) and GacS (21) control formation of biofilms. Production of the biofilm depends on pili which are assembled by the chaperone-usher system (CsuABCDE) (22) and the biofilm-associated protein (BAP) (23). In addition, a four gene locus, *pgaABCD*, encodes enzymes required for the synthesis of poly-beta-1-6-*N*-acetylglucosamine, an

extracellular polysaccharide that forms the biofilm matrix and supports the typical three-dimensional architecture of biofilm (24). A general *O*-linked glycosylation system encoded by *pglL* is required for the glycosylation of several membrane proteins. PglL is an O-oligosaccharyltransferases, and *pglL* mutant strains make less biofilms, are less virulent in the *Dictyostelium discoideum* and *Galleria mellonella* models of infection, and are less competitive in BALB/c mice when compared to the wild-type (25). Understanding more about how *A. baumannii* produces and maintains its biofilms may reveal new strategies for controlling its spread in hospital settings.

In addition to surviving on surfaces, many strains of *A. baumannii* survive in serum and withstand complement-mediated killing. Part of this resilience is due to capsular polysaccharides (26). The genes in the sequence variable cluster or K locus determine which polysaccharides are displayed on the surface, thus affecting capsule composition and structure (14, 26). An additional mechanism may be the production of a plasminogen binding protein, CipA. Once bound to CipA, the plasminogen degrades fibrinogen and the complement protein C3b (27). Lack of CipA decreases survival in serum, specifically through the alternative complement pathway (27). Additional mechanisms include cell-surface binding of Factor H (28, 29) and complement inactivation by the serine protease PKF (30). Another contributor to growth in serum is phospholipase D (PLD) (31). There are three different genes which code for PLD, and, in addition to supporting growth in serum, they appear to work in concert for epithelial cell invasion (32). Phospholipase C (PLC) and PLD are also important in iron acquisition. By cleaving the phospholipids in host membranes, the phospholipases initiate cytolysis of the host cells. The release of host intracellular contents after lysis

can release iron (33). This lysis can be membrane specific. For example, *A. baumannii* is able to lyse sheep, human, and horse erythrocytes but to varying degrees. The abundance of erythrocyte phosphatidylcholine present in the membrane is positively correlated with cell lysis (34). The combination of all these proteins and systems supports the survival of *A. baumannii* in a hostile environment and allow it to colonize.

Another route of survival is to affect the host cells using outer membrane vesicles (OMV). OMVs are used as delivery vehicles that transport effector proteins to host cells, allowing for interaction between the bacterium and the host cell without close physical contact. The OMVs of *A. baumannii* contain a wide variety of effectors, including OmpA, proteases, phospholipases, superoxide dismutase, and catalase (35). OmpA, a porin, is necessary for bronchial epithelial cell invasion and cytotoxicity (36, 37). Another OMV protein, a porin Omp 33-36, also induces cytotoxicity by activating caspases which lead to autophagy of the mammalian cell (38). This cytotoxic effect could be targeting cells like macrophages as a mechanism to evade the immune system (38). *A. baumannii* OMVs also induce an inflammatory response by upregulating cytokine genes in the lung after intratracheal injection of OMVs (39). While OMVs are not unique to *A. baumannii*, they do provide a vast amount of benefits to the bacterium.

Like other pathogenic bacteria, *A. baumannii* must possess a way to thrive in the human body where there is a lack of free iron (40). One strategy is to produce a siderophore, acinetobactin (41, 42). While many strains do have the genes to produce this siderophore, others are able to acquire iron without it, suggesting that *A. baumannii* strains possess and utilize different iron-acquisition pathways (43, 44). Another possible

method of iron acquisition is the formation of iron-sulfur (Fe-S) proteins, which can store iron and thereby act as an iron pool. Eukaryotes and prokaryotes have developed systems to regulate the formation of such proteins, as spontaneous formation would only occur under toxic levels of iron and sulfur. Fe-S proteins are necessary for *A. baumannii* survival; lack of one of the scaffold Fe-S proteins, NfuA, results in an inability to persist in human epithelial cells and the inability to kill *Galleria mellonella* larvae (45).

Just as free iron is tightly controlled in the human body but highly necessary for bacterial survival, so are other transition metals such as zinc and manganese. While no mechanism for manganese acquisition has been discovered to date, a zinc-acquisition system called Znu has been identified in *A. baumannii* (46). This system is an ABC transporter and is required for full colonization of the lung in a murine pulmonary model of infection wherein mutant strains are outcompeted by the wild-type strain (46). By deploying systems to acquire metals, *A. baumannii* is more competitive in a nutrient-deficient environment.

While there is a plethora of virulence and colonization factors described in *A. baumannii*, we still do not have a complete understanding of its pathogenesis. Thus, we are at a great disadvantage when it comes to treating outbreaks and designing therapeutics for antibiotic resistant *A. baumannii* as we are still missing the full picture.

Type II Secretion System

Many Gram-negative bacteria transport virulence factors across the cell envelope using secretion systems, such as the Type II Secretion System (T2SS). One of seven

protein secretion systems used by Gram-negative bacteria (47), the T2SS is most commonly found among members of the Proteobacteria, including *Vibrio cholerae,* Enterotoxigenic and Enterohemorrhagic *Escherichia coli, Pseudomonas aeruginosa* and *Legionella pneumophila* (48, 49) (50). The T2SS is a multiprotein complex that is responsible for extracellular secretion of toxins, proteases, lipases, and enzymes that break down complex carbohydrates (a small subset of these are shown in Table 1.1). It is encoded by between 12 to 16 general secretion pathway (*gsp*) genes (Figure 1.1) (48, 49, 51) and spans the inner and outer membranes.

The *gsp* genes are named *gspA* through *gspO* and *gspS*, with *gspC* though *gspM* being essential for T2S. While many of the bacterial species that carry the T2S genes use the *gsp* notation, there are some deviations such as *eps* in *V. cholerae* and *xcp* in *P. aeruginosa*. If any of the genes are missing, the secretion apparatus is no longer functional (52).

The inner platform of the secretion apparatus is composed of GspE, GspF, GspL, and GspM (53). GspE is the secretion ATPase, which powers the secretion apparatus and resides in the cytoplasm (54, 55). GspE is attached to this platform via interaction of its N-terminus with GspL (55, 56). GspL and GspM stabilize each other and protect each other from degradation (57). GspL also interacts with the major pseudopilin, GspG, and may serve as the means to transport energy from the ATPase to the pseudopilus (58). The last member of this platform is GspF, which, like the other members, plays a role in stabilization (53).

GspC is anchored to the inner membrane and exposed to the periplasmic space. Studies have demonstrated an interaction between GspC and the outer membrane porin, GspD (59). GspC has two important structural domains: the PDZ domain and homology region (60). The PDZ domain in GspC and the N-terminal domain of GspD are important determinants for T2S substrate specificity (61, 62). While the PDZ domain is necessary for secretion of some proteins, it is not necessary for all. Some GspC have a coil-coiled domain in place of the PDZ domain which plays a similar role as the PDZ domain (63). The homology region interacts directly with GspD. GspD forms the gated outer membrane porin or secretin by forming a pentadecameric structure (64).

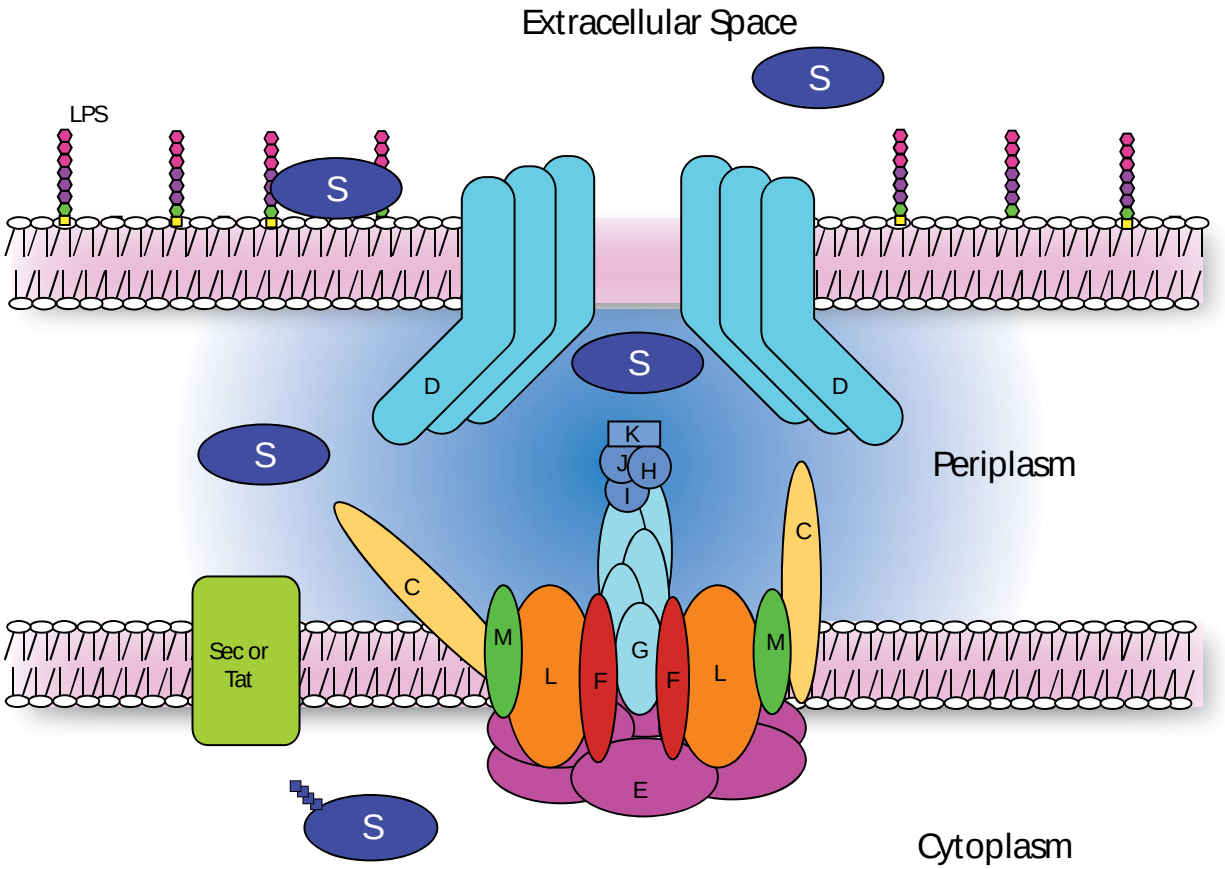

Figure 1.1. A schematic representation of the T2S System in *A. baumannii*. The T2S apparatus spans the inner membrane, periplasm, and outer membrane. T2S substrates (indicated by S) are transported through the Sec or Tat system, fold in the periplasm, and are secreted out through the outer membrane porin (GspD). Once secreted, the substrates may be cell-associated or fully secreted. GspG, H, I, J, and K form the pseudopilus while GspC, M, L, and F make up the inner platform. GspE is an ATPase, which powers the system. S, substrate.

GspG, H, I, J, and K make up the pseudopilus, so named because it is not present on the bacterial surface but is similar to the Type IV pilus (Figure 1.1). GspG is called the major pilin as it is the most abundant of the pilins while GspH, I, J, and K are the minor pilins. The prepilin peptidase, GspO, is responsible for the maturation of the pseudopilins by cleavage and methylation(65). GspI has been implicated in initiation of pseudopilus assembly, as there is a reduction in the number of pili in a *gspI* mutant (66). GspK is localized to the tip of the pseudopilus (67). It is not currently known how the pseudopilus transports substrates to the outer porin, GspD. One hypothesis is a piston-

like model that envisions the pseudopilus forming to push a substrate out of the outer porin and then retracting to pick up the next substrate (67). However, unlike in the similarly constructed Type IVa pilus, there is no retraction ATPase for the T2S, so the question of how the piston would retract remains to be answered. Studies have shown that GspK destabilizes GspG and could cause the pseudopilus to degrade, which could explain how the pseudopilus separates from GspD (66). However, this mechanism would necessitate reassembly of the pseudopilus for every substrate. Another theory is an Archimedes screw-like model wherein substrates continually attach to the pseudopilus for transport to GspD on an ever present escalator (68).

Proteins that are T2S substrates have an N-terminal signal sequence that targets them to either the Sec or Tat export systems (69). Once in the periplasmic space, the proteins fold and interact with GspC, GspD, and the pseudopilus, suggesting that the substrates are recruited by GspC. The exact mechanism of this recruitment is unknown. However, a study using the T2S substrate PulA from *K. oxytoca* showed that a three dimensional structural motif may be required for secretion, indicating that identification of a T2S signal may require an evaluation of structural motifs in the folded protein rather than amino acid sequences alone (70). Regardless of the exact recruitment mechanism, the substrates then bind to the pseudopilus and are secreted out through GspD (63).

Four additional Gsp proteins are present in some but not all bacteria with a T2SS: GspA, B, N, and S. GspA and GspB aid in the assembly of the secretin. However, even in bacterial species where they are present, they may not be required for secretion, suggesting they may have a redundant role in those species or they may

only be required under certain growth conditions (71). Similarly, GspN also binds to GspD and may play a role in stabilization. Just as with GspA and B, it is also not required for secretion in all the bacterial species where it is present (72). GspS is a lipoprotein which is located in the outer membrane and aids in the insertion and stabilization of GspD (73, 74). These four proteins may play more species specific roles rather than global T2S roles.

Table 1.1. Examples of T2S system substrates.

Bacterial Species	**Substrates**	**References**
V. cholerae	Cholera Toxin, Serine proteases, Lipase	(49, 75, 76)
E. coli (ETEC)	Metalloprotease, Heat-labile toxin	(77, 78)
E. coli (EPEC)	Lipoprotein	(79)
E. coli (EHEC)	Metalloprotease	(80)
Pseudomonas aeruginosa	Lipases, Alkaline Phosphatase	(81, 82)
Klebsiella oxytoca	Pullulanase	(83)
Legionella pneumophila	RNase, lipase, phospholipase, chitinase	(84, 85)

Host Response to Bacterial Infections

Just as bacteria have evolved many mechanisms, such as secretion systems, to survive in their respective environments, the host has also developed systems to detect and destroy bacteria. While a full discussion of the interaction between bacteria and the host is outside the scope of this dissertation, I will provide some examples. Bacteria must get past innate immune defenses, including breaching physical barriers. Many immune cells have pattern recognition receptors that have evolved to detect invaders.

For instance, host cells possess Toll-like receptor 4 which recognizes bacterial lipopolysaccharide (LPS) present on the outer membrane of Gram negative bacteria and upregulates immune effector cells (86). Another important aspect of the immune system is the complement pathway, which some researchers have hypothesized constitutes a link between the innate and adaptive immune system (87). If a pathogen enters the blood, it must have some way of escaping the three arms of complement: classical, alternative, and lectin pathways as described above (page 5). In addition to the previously mentioned examples of complement evasion is the production of a serine protease, Pic, by both *Shigella flexneri* and *E. coli* O104:H4. This protease cleaves a common component of complement and contributes to immune evasion by these two pathogens (88). In addition to the complement system, pathogens in the blood must also contend with the coagulation system described in the next section.

Coagulation Pathway

In the vast majority of people, the blood hemostasis system responds to lacerations by forming a clot to stop the bleeding in a process called coagulation. Coagulation is tightly controlled to form clots only when needed, thus preventing potential damage caused by aberrant clotting, thrombosis, that may lead to stroke or heart attacks. This complex process is depicted by the coagulation cascade (Figure 1.2).

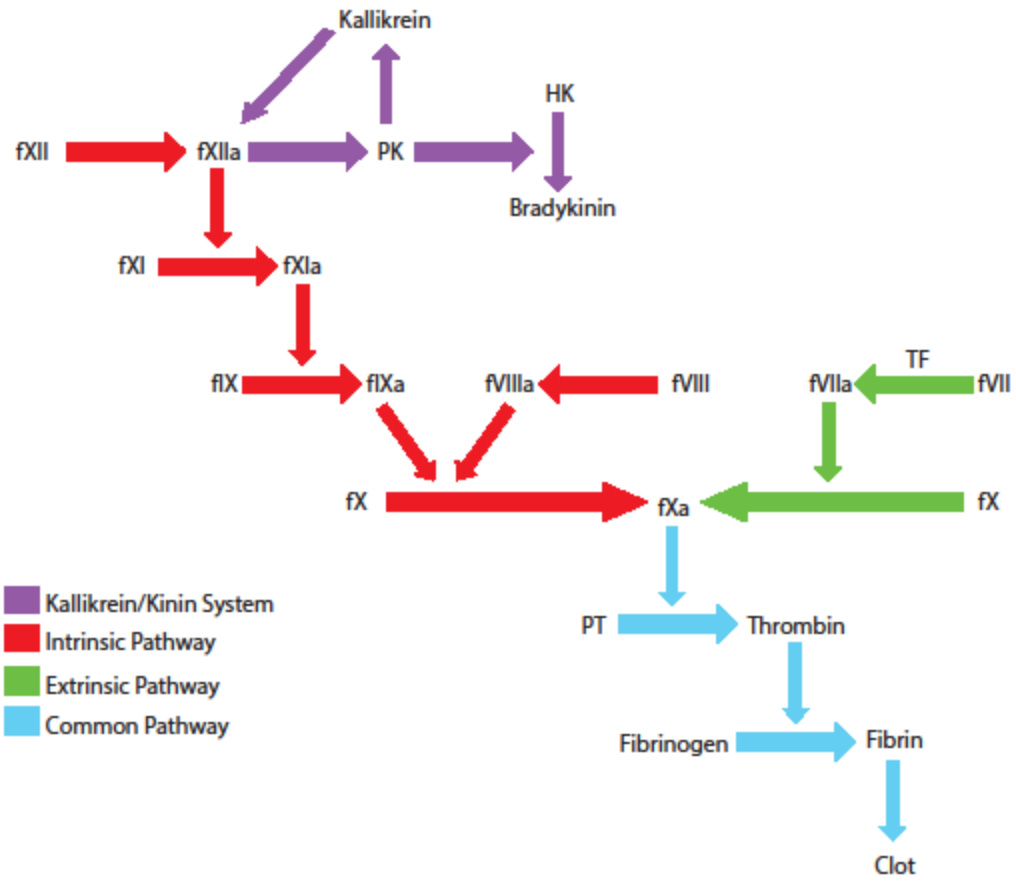

Figure 1.2. A simplified coagulation cascade. PT, prothrombin; f, Factor; TF, tissue factor; PK, prekallikrein, HK, high-molecular weight kininogen.

The coagulation cascade was initially described as a waterfall sequence with many of the coagulation factors existing as inactive precursor enzymes or zymogens (89). Once activated, they go on to cleave the next zymogen, until fibrinogen is cleaved and a fibrin clot is formed (Figure 1.2). In addition, regulatory proteins, including inhibitors, target many steps of the pathway ensuring that clotting only occurs under the correct circumstances. Thrombosis can occur when a repressive protein is down-regulated or an activator is upregulated, causing intravascular clots to form unchecked. The coagulation cascade can be divided into two pathways: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is also known as the contact-dependent

pathway while the extrinsic pathway is known as tissue-dependent. The significance of the demarcation of these two branches may not be apparent *in vivo*, but it is useful during clinical testing.

Factor XII (fXII), also known as Hageman factor (90), is the beginning of the intrinsic pathway of the coagulation cascade. When fXII comes into contact with a negatively-charged surface, it autoactivates into fXIIa and starts the cascade (91). The rest of the intrinsic pathway is made up of factor XI (fXI), factor IX (fIX), and factor VIII (fVIII). The activated partial thromboplastin time (aPTT) assay is used to measure the function of the intrinsic pathway. A negatively charged substance, like kaolin, is added to the plasma to activate fXII and clotting time is measured.

In contrast, the extrinsic pathway starts by the binding of factor VII (fVII) to its receptor, tissue factor (TF). The majority of TF is located beneath the endothelial layer and, thus, cannot be accessed by fVII without a physical disruption of the endothelium. Additionally, high levels of TF can be found in particular organs like the brain, heart, lung, uterus, placenta, and testes (92). One possible explanation for this distribution is the high amount of damage that could take place if bleeding occurred in any of these organs. The prothrombin time (PT) assay, which measures the function of the extrinsic pathway, is conducted by adding exogenous TF to the plasma and measuring clot time. Both branches eventually meet at factor X (fX) of the common pathway, as components of both the intrinsic and extrinsic pathways cleave fX into fXa (Figure 1.2).

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Clotting and Pathogenesis

In addition to preventing loss of blood, clots can also aid the immune system by effectively trapping pathogens that enter the bloodstream. Some pathogens can combat the effects of coagulation by targeting different effectors in the coagulation cascade. All pathogenic group A streptococci encode the protein streptokinase, which activates plasminogen (93) (an anti-clotting agent), as do staphylokinase from *Staphylococcus aureus* (94) and Pla protease from *Yersina pestis* (95). By activating plasminogen, the bacteria counteract the formation of localized clots (96) which would otherwise trap the bacteria and prevent dissemination.

Fibrin, the main component of clots, is also necessary for the formation of abscesses which can trap bacteria (97). Another bacterium, *Aeromonas sobria,* secretes a serine protease which cleaves fibrinogen (98). As with the plasminogen activators, this enzyme decreases clot formation. Neutrophil extracellular traps (NETS) could provide a link between bacterial infections and thrombosis (99). When NETS entrap a bacterium, they may stimulate clot formation by providing a scaffold for fibrin polymerization (99).

In addition to preventing clots, some bacteria may also evade clots altogether. *Streptococcus pyogenes* expresses a protein (M-protein) that, in addition to preventing phagocytosis and protecting from the killing action of complement (100), captures and cleaves high molecular weight kininogen (HK), increasing the generation of bradykinin (101). By utilizing the vascular permeability inducing properties of bradykinin, *S. pyogenes* may then increase its dissemination throughout the body (101). For bacteria

that enter through the blood stream, having a method to circumvent the action of the coagulation system and the impermeability of the blood vessels helps greatly in their survival and dissemination.

Factor XII

Lack of a coagulation factor can have dire consequences. While the absence of some factors make a fetus unviable, loss of others can lead to a serious bleeding disorder, such as hemophilia. FXII-deficiency does not result in a bleeding disorder even with an increase in clotting time, as measured by an aPTT assay; however recent studies have suggested that loss of fXII may reduce the development of thrombi or clots *in vivo*, leading to reduced rates of heart attacks and strokes. Specifically, mice lacking fXII, like humans, have normal bleeding time and display no spontaneous bleeding *in vivo*. However, the fXII-deficient mice are deficient in thrombus formation, thereby preventing the generation of life-threatening thrombi in a process that can be reversed by adding exogenous human fXII (102). These observations in mice lead to the hypothesis that fXII may not be essential for forming clots in response to damaged blood vessels but instead be required for forming clots due to intrinsic factors such as the presence of a pathogen.

The importance of fXII is not limited to the coagulation cascade. In addition to activating the intrinsic pathway, fXII also activates C1 esterase, a component of the classic complement cascade (not shown), and converts prekallikrein into kallikrein (see Figure 1.2). Kallikrein can in turn activate more fXII as well as liberate bradykinin from HK as mentioned above (Figure 1.2 and references (103, 104), increasing vascular

permeability. Kallikrein can also activate other members of the complement cascade, C3 and C5 (105, 106). Given the many different pathways involving fXII, it seems unlikely that it is merely a redundant protein and has no *in vivo* significance, as some have suggested.

Mutations in fXII can lead to dysregulation of bradykinin production. One such mutation is the missense mutations T309K (107) and T309R of fXII (108). These familial mutations cause hereditary angioedema type III, which involves swelling of subcutaneous tissue due to leakage of intravascular fluid. This subgroup of angioedema has normal C1-esterase inhibitor suggesting that the mutation in fXII is the cause. The T309K and T309R substitutions disrupt *O*-glycosylation leading to an increase in fXII contact activation and generation of bradykinin, followed by the tell-tale sign of angioedema, or swelling (109).

Scope of this Study

My work aims to examine the pathogenesis of *A. baumannii* and determine how its T2S system can be instrumental in this process. Chapter two describes the functional characterization of the T2S system in *A. baumannii* and one of its substrates, LipA. We use a murine bacteremia model to demonstrate the importance of the T2S system and LipA in colonization. In chapter three, we investigate another T2S substrate, CpaA, and describe the activity of CpaA against the coagulation factor fXII. This work highlights the importance of fXII glycosylation for the cleavage by CpaA. In chapter four, I describe the development of a high-throughput screen to identify small molecule inhibitors of the T2S system. We optimize a lipase assay as well as develop counterscreens to aid in

removing false positives. This screen opens up the possibility of not only identifying inhibitors against the T2S system in *A. baumannii* but also general T2S inhibitors for targeting other pathogens. Compounds may be developed into a drug to combat antibiotic-resistant pathogens or may be used in the laboratory to study the T2S system in multi-drug resistant strains.

Altogether, my work provides insight into the pathogenesis of *A. baumannii*. We gain a deeper understanding of how the T2S system may promote bacterial survival in the host. To help combat *A. baumannii* pathogenesis, we have developed a workable screen for the identification of T2SS inhibitors.

Chapter II:

Acinetobacter baumannii is Dependent on the Type II Secretion System and Its Substrate LipA for Lipid Utilization and *in vivo* Fitness

Modified from:

Johnson TL, Waack U, Smith S, Mobley H, Sandkvist M. 2016. *Acinetobacter baumannii* is dependent on the type II secretion system and its substrate LipA for lipid utilization and *in vivo* fitness. J Bacteriol 198:711–719.

Abstract

Gram-negative bacteria express a number of sophisticated secretion systems to transport virulence factors across the cell envelope, including the type II secretion (T2S) system. Genes for the T2S components GspC through GspN and PilD are conserved among isolates of *Acinetobacter baumannii*, an increasingly common nosocomial pathogen that is developing multi-drug resistance at an alarming rate. In contrast to most species, however, the T2S genes are dispersed throughout the genome rather than linked into one or two operons. Despite this unique genetic organization, we show here that the *A. baumannii* T2S system is functional. Deletion of *gspD* or *gpsE* in *A. baumannii* ATCC 17978 results in loss of secretion of LipA, a lipase that catabolizes long-chain fatty acids. Due to lack of extracellular lipase, neither the *gspD* mutant, *gspE* mutant or a *lipA* deletion strain grows on long-chain fatty acids as a sole

source of carbon, while their growth is indistinguishable from that of the wild-type strain in nutrient rich broth. Genetic inactivation of the T2S system and its substrate LipA also reduces *in vivo* fitness in a neutropenic murine model for bacteremia. Both the *gspD* and *lipA* mutants are outcompeted by the wild-type strain, as judged by their reduced numbers in spleen and liver following intravenous co-inoculation. Collectively, our findings suggest that the T2S system promotes *in vivo* survival of *A. baumannii* by transporting a lipase that may contribute to fatty acid metabolism.

Importance

Infections by multi-drug resistant *Acinetobacter baumannii* are a growing health concern worldwide, underscoring the need for a better understanding of the molecular mechanisms by which this pathogen causes disease. In this study we demonstrate that *A. baumannii* expresses a functional type II secretion (T2S) system that is responsible for secretion of LipA, an extracellular lipase required for utilization of exogenously added lipids. The T2S system and the secreted lipase support *in vivo* colonization and thus contribute to the pathogenic potential of *A. baumannii.*

Introduction

Acinetobacter baumannii, an increasingly common nosocomial Gram-negative pathogen, is responsible for a wide range of infections, including pneumonia, urinary tract infections, bacteremia, meningitis, and skin and wound infections (1-3, 9). Immunocompromised and severely ill patients in the intensive care unit, individuals with extensive wounds or invasive devices, and those undergoing or having recently undergone antibiotic regimens are particularly susceptible to *A. baumannii* infections (4-

6). Ventilator-associated pneumonia and blood stream infections are the most severe, resulting in 25-35% mortality rates (110, 111).

The pathogenic success of *A. baumannii* is likely multifactorial, but of importance are its ability to persist on dry surfaces, form biofilm, resist complement-mediated killing and survive antibiotic treatment (1, 26, 112-114). The escalating frequency of multidrug-resistant (MDR) strains of *A. baumannii* is of particular concern. In the past ten years there has been an alarming 60% increase in reported MDR clinical isolates (http://www.cddep.org). An important and clinically relevant aspect of bacterial infections is the ability of bacteria to grow as biofilm (115). These matrix-encased, multi-layer bacterial communities are exceptionally resistant to antibiotic treatment and are prone to spreading antibiotic resistance through horizontal gene transfer (116, 117) . Clinical isolates that form biofilms survive for long periods of time on dry surfaces and are able to colonize common hospital equipment, such as ventilator tubes (118). Several factors are necessary for abiotic biofilm formation, including a pilus assembly system, the outer membrane protein OmpA, and capsular polysaccharide, which is also protective against complement-mediated killing (24, 26, 37, 119).

While research has focused on the mechanisms of antibiotic resistance, biofilm formation and the epidemiology of *A. baumannii*, our understanding of *A. baumannii* pathogenesis is lagging and little is known about the contribution of secreted proteins to *A. baumannii* survival and propagation during infection. Sequencing of several *A. baumannii* genomes revealed that *A. baumannii* contains genes for a variety of transport systems, including the assembly and translocation system for Type IV pilus and type IV and type VI secretion systems. The Type IV pilus supports twitching motility

of *A. baumannii* (120), but may also contribute to adhesion, colonization, biofilm formation and transformation like type IV pili in other Gram-negative pathogens, while the type IV secretion system and type VI secretion system are required for virulence and bacterial competition, respectively (121, 122). In addition, *A. baumannii* possesses genes for a type II secretion (T2S) system (47, 123). Bacteria that use the T2S system typically reside in the environment; however, they also include pathogens such as *Vibrio cholerae,* Enterotoxigenic *Escherichia coli, Pseudomonas aeruginosa*, and *Legionella pneumophila* (48, 50, 124, 125). The T2S system mediates the secretion of toxins and hydrolytic enzymes, including proteases, lipases, lipoproteins and enzymes that break down complex carbohydrates, and is required for *in vivo* survival and virulence (75, 78, 79, 85, 126-131). Following inner membrane translocation via the Sec or TAT pathways, T2S substrates engage with the T2S system for transport across the outer membrane. This multiprotein secretion system is encoded by 12 to 16 general secretion pathway (*gsp*) genes (68, 132, 133). With rare exceptions, mutations in any of the core *gsp* genes, *gspC* through *gspM* and *pilD*, prevent extracellular secretion (133).

The *gsp* genes are scattered throughout the *A. baumannii* genome instead of being organized into one or two operons (48). Due to their unusual arrangement, it was unclear whether the *gsp* genes of *A. baumannii* encode a functional secretion system. Here, we demonstrate that the T2S system in *A. baumannii* is functional and identify a lipase as one of its secreted substrates. We show that the extracellular lipase, LipA, as well as the T2S system that transports this enzyme across the outer membrane are required by *A. baumannii* to utilize exogenously added lipids and support colonization of a mouse in a model of bacteremia.

Materials and Methods

Bacterial Strains and Growth Conditions

All strains listed in Table 2.1 were cultured in Luria-Bertani (LB) broth or on LB agar at 37° C. Carbenicillin (100 µg/ml) was used for plasmid maintenance.

Construction of Δ*lipA*, Δ*gspD*, Δ*gspE1*, Δ*gspE2*, and Δ*gspN* strains

Chromosomal DNA isolated from the wild-type (WT) *A. baumannii* ATCC 17978 strain was used as the template for polymerase chain reaction (PCR). PCR reactions were carried out with Phusion DNA polymerase. Primers were synthesized by IDT Technologies.

To generate the Δ*gspD* strain, we used the primers indicated in Table 2.2 to amplify 500-bp DNA upstream and downstream of the *gspD* gene as well as the *aph-3* kanamycin cassette and cloned into pCVD442. Transconjugates in which pCVD442 had recombined into the *A. baumannii* genome were selected on LB agar containing carbenicillin and chloramphenicol. To select for the second recombination event, individual colonies were cultured overnight in LB broth, diluted, cultured to late log phase, and spread on LB agar containing 3% sucrose. Sucrose and kanamycin-resistant, carbenicillin-sensitive isolates were screened for loss of growth on lipid agar. Strains that were kanamycin-resistant and carbenicillin-sensitive were designated Δ*gspD*. The deletion was verified by PCR. All other gene deletion strains (Δ*lipA*, Δ*gspE1*, Δ*gspE2*, and Δ*gspN*) were constructed in a similar manner using the appropriate primers shown in Table 2.2.

Construction of p*lipBA,* p*lipA*, p*gpsE1*, p*gspE2*, p*gspN*, and p*gspD* plasmids

The *lipA* and *lipB* genes were amplified from chromosomal DNA using the appropriate primers shown in Table 2.2. The product was ligated into a low-copy, broad-host vector pMMB67EH to make p*lipBA*. This broad-host expression vector has been used in many gram-negative species including *P. aeruginosa* and *V. cholerae* (134) and is stably maintained in *A. baumannii*. The construct was verified by sequencing and conjugated from the *E. coli* strain MC1061 into WT and mutant *A. baumannii* strains. The plasmids overexpressing *lipA, gspD*, *gspE1*, *gspE2*, and *gspN* were constructed the same manner.

Lipid Agar

Selective agar was utilized to detect extracellular lipase activity. The medium was prepared as described previously (135) with modified minimal medium (47.8 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 18.7 mM NH_4Cl_2, 0.1% Tween 20, 0.2 mM $CaCl_2$), 40 µg/mL of neutral red and 0.5% filtered-sterilized olive oil.

Lipase Assay

The strains were grown in LB broth supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) at a 50 µM final concentration to induce the expression of plasmid-encoded LipA. Following 16 hr growth, supernatants and cells were separated by centrifugation at 3500 rpm for 10 min. A spectrophotometric assay was used to measure lipase activity by incubating culture supernatant with 0.9 mM *p*-nitrophenyl myristate in 80 mM Tris-HCl, pH 8.0 and 0.15% Triton X-100 buffer at 37° C and

measuring the release of *p*-nitrophenol at 415 nm over time. All assays were performed in triplicate and means and standard deviation are presented.

SDS-PAGE and Immunoblotting

Culture supernatants were concentrated by precipitation utilizing pyrogallol red-molybdate-methanol as described previously (75). The samples were normalized to equivalent optical density at 600 nm and subjected to SDS-PAGE and immunoblot analysis using antibodies raised against *A. calcoaceticus* LipA (1:100) (136) and goat anti-rabbit IgG-HRP. Immunoblots were imaged using a Typhoon Trio (Amersham Biosciences).

Serine Hydrolase Probe

Overnight cultures of WT/p*lipBA* and Δ*gspD*/p*lipBA* were cultured in LB broth with 50 µM IPTG at 37°C. Supernatants and cells were separated by centrifugation at 3500 rpm for 10 min. ActiveX^{TM} FP Serine Hydrolase probe (0.5 µL) (Thermo Scientific) was added to 25 µL culture supernatants and incubated at room temperature for 60 min. Samples were matched by equivalent OD_{600}, boiled in SDS sample buffer, subjected to SDS-PAGE on 4 to 12% Bis-Tris polyacrylamide gels (NuPAGE; Invitrogen), and visualized using a Typhoon Trio variable mode imager system and ImageQuant software.

***In vitro* Competition Assay**

WT and mutant *A. baumannii* strains were cultured separately overnight in LB broth at 37°C. Strains were diluted 1:100 and equivalent numbers of WT and mutant strains were pooled and cultured together at 37°C. At 0 (input), 8, and 24 hr, aliquots of

the mixed culture were diluted and plated on to LB agar with and without kanamycin. CFU counts were determined after 24 hr incubation at 37° C. The competitive index (CI) was determined after 24 hours. CI = ([mutant CFU/WT CFU]/[mutant Input CFU/WT Input CFU]).

In vivo competition assay

Eight-week old female CBA/J mice (Jackson Laboratory) were injected intravenously with 150 µL and 100 µL of 20 mg/mL cyclophosphamide four and three days, respectively, before the start of the experiment. Overnight cultures of WT, *ΔlipA,* and Δ*gspD A. baumannii* strains were diluted in phosphate buffered saline (PBS). Inocula of 10^7 cells of 1:1 ratios of WT:Δ*lipA* or WT:Δ*gspD* were administered via tail-vein injection. After 24 hr, mice were euthanized. Spleens and livers were removed and homogenized in PBS and CFU counts were determined after plating on LB with and without kanamycin and 24 hr incubation at 37° C. CI was calculated as described above.

Statistical Tests

A Wilcoxon Signed Ranked test was calculated for the CIs obtained for the *in vitro* and *in vivo* competition assays. A student *t*-test was calculated for the lipase activity assay. Values were considered significant at $P \leq 0.05$.

Ethics statement

All mice experiments were performed according to the protocol (PRO00005052) approved by the University Committee on Use and Care of Animals at the University of

Michigan. This protocol is in complete compliance with the guidelines for humane use and care of laboratory animals mandated by the National Institutes of Health.

Results

***A. baumannii* encodes a functional T2S system.** We analyzed the sequenced genome of several *A. baumannii* strains and performed homology searches with the T2S genes of *V. cholerae* and *P. aeruginosa*. We identified genes for each of the T2S components GspC through GspN and PilD (Figure 2.1).

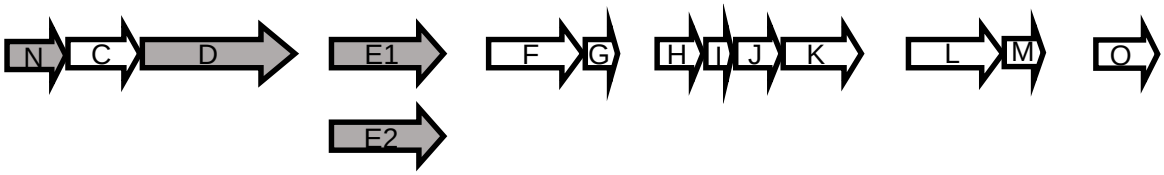


Figure 2.1. T2S genes in *A. baumannii*. Putative T2S components are encoded by gspN (A1S_0269), gspC (A1S_0270), gspD (A1S_0271), gspE1 (A1S_0616), gspE2 (A1S_2290), gspF (A1S_0369), gspG (A1S_0370), gspH (A1S_1562), gspI (A1S_1563), gspJ (A1S_1564), gspK (A1S_1565), gspL (A1S_2255), gspM (A1S_2254) and gpsO (also named pilD; A1S_0327). The genes shown in grey were deleted using allelic exchange technology.

In contrast to most species, these genes are scattered around the genome in six non-contiguous segments rather than linked into one or two operons, perhaps as a result of genome plasticity and the remarkable ability of *A. baumannii* to acquire foreign DNA. To determine whether *A. baumannii* has a functional T2S system, we utilized *A. baumannii* ATCC 17978, a strain that was originally isolated from a 4 month old with fatal meningitis and is amenable to genetic inactivation and plasmid-based complementation studies. Using allelic exchange, we inserted a kanamycin resistance gene cassette in place of *gspD, gspE1, gspE2,* or *gspN*. The *gspD* gene was chosen because in all studied T2S systems, GspD, the outer membrane pore that serves as the conduit through which proteins are transported, is absolutely essential for T2S. GspE

contributes energy for the secretion process by hydrolyzing ATP and is also indispensable for secretion. However, as *A. baumannii* carries two potential *gpsE* genes, *gspE1* and *gspE2*, we deleted each gene to resolve which *gspE* gene (or both) is required for secretion in *A. baumannii.* In contrast to GspD and GspE, the role of GspN has not yet been determined, and its gene is not present in every species with a functional T2S system. As *gspN* is localized in the same operon as *gspD* in *A. baumannii*, we wanted to determine whether it is also required for T2S in *A. baumannii*. To complement each deletion strain, we constructed expression vectors encoding the wild-type (WT) copy of each mutant gene and expressed them *in trans*.

While the Δ*gspD* and Δ*gspE1* mutants grew on LB agar as well as the WT, Δ*gspE2*, and Δ*gspN* strains, they were unable to grow on minimal agar with olive oil as the sole carbon source (Figure 2.2A), a phenotype previously observed for T2S mutants of *P. aeruginosa* and *V. cholerae* due to their inability to secrete lipase (126, 135). Growth was restored when either the Δ*gspD* or Δ*gspE1* mutants were complemented with the appropriate expression plasmids (Figure 2.2A).

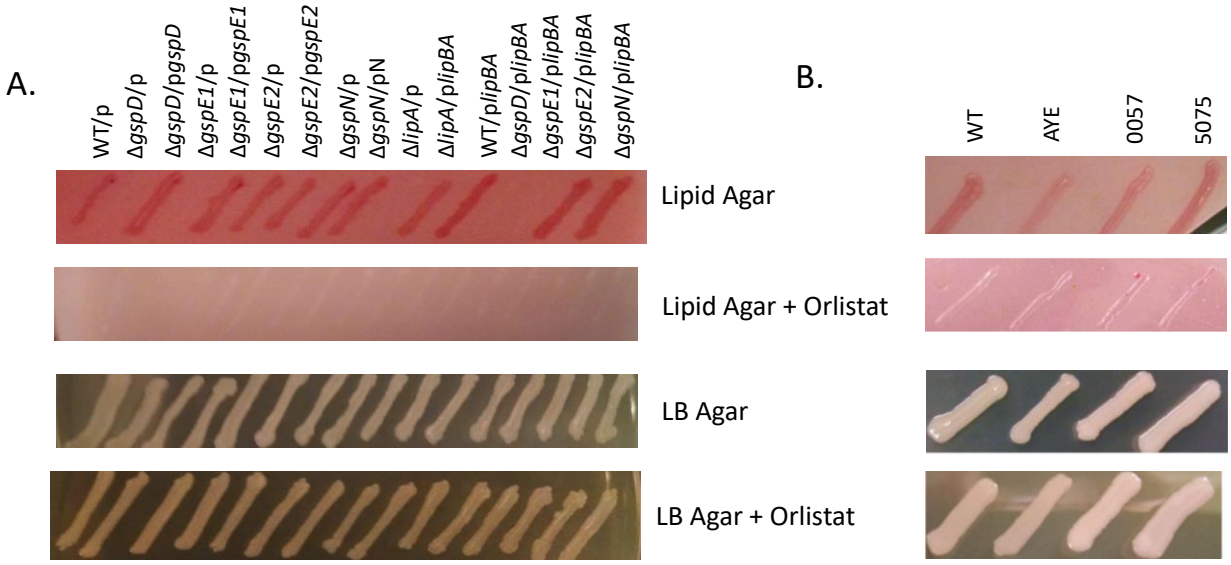


Figure 2.2. *A. baumannii* growth on lipid agar and LB. Growth of strains, from top to bottom, on lipid agar, lipid agar with orlistat, LB agar, and LB agar with orlistat. A. Agar included carbenicillin and IPTG for plasmid maintenance and induction of expression of cloned genes. B. Growth of reference strain ATCC 17978 (WT) and three clinical isolates.

Sequence analysis of *A. baumannii* ATCC 17978 identified genes with high homology to the genes of the *P. aeruginosa* and *V. cholerae* T2S substrate LipA (Figure 2.3) and its chaperone LipB (76), suggesting that the *A. baumannii* LipA may also be a T2S substrate that is capable of hydrolyzing lipids and generating nutrients for growth. To verify that LipA is the secreted substrate responsible for growth of *A. baumannii* on lipid agar, we constructed a *lipA* deletion strain by replacing the *lipA* gene with a gene encoding kanamycin resistance through homologous recombination. Similarly to the Δ*gspD* and Δ*gspE1* mutants, the Δ*lipA* strain was unable to grow on the minimal lipid agar (Figure 2.2A). Growth was restored when the *lipA* mutant was complemented with a plasmid encoding *lipA* and *lipB*. We also expressed the plasmid-encoded *lipBA* genes in the Δ*gspD* and Δ*gspE1* deletion strain; however, despite overexpression of *lipBA*, no

growth was observed on lipid agar (Figure 2.2A). This suggests that the T2S system is active in *A. baumannii* and is responsible for the extracellular secretion of LipA.

Figure 2.3. Sequence alignment of LipA. The alignment of LipA from *A. baumannii*, *V. cholerae*, and *P. aeruginosa*. Predicted N-terminal signal sequence is underlined. Yellow highlights indicate the catalytic residues.

When *A. baumannii* is cultured in the absence of lipids in LB broth under standard laboratory conditions, the *lipBA* genes are likely not expressed. This is consistent with our inability to detect significant differences in lipase activity between culture supernatants of WT, Δ*gspD*, Δ*gspE1*, Δ*gspE2*, Δ*gspN*, and Δ*lipA* strains (Figure 2.4). Therefore, to quantify the level of LipA secretion, we induced expression of plasmid-encoded *lipBA* genes in the WT, Δ*gspD*, Δ*gspE1*, Δ*gspE2*, and Δ*gspN* strains. This resulted in detectable lipase activity in the culture supernatant of WT/p*lipBA*, Δ*gspE2*/p*lipBA*, and Δ*gspN*/p*lipBA* using 4-nitrophenyl myristate, while no activity was

observed in the Δ*gpsD*/p*lipBA* and Δ*gspE1*/p*lipBA* culture supernatants (Figure 2.4A). In addition, immunoblotting with antibodies directed against *A. calcoaceticus* LipA detected extracellular LipA when overexpressed in WT *A. baumannii* but not in the Δ*gspD* mutant, providing further support for the requirement of a functional T2S system for extracellular secretion of LipA (Figure 2.4B).

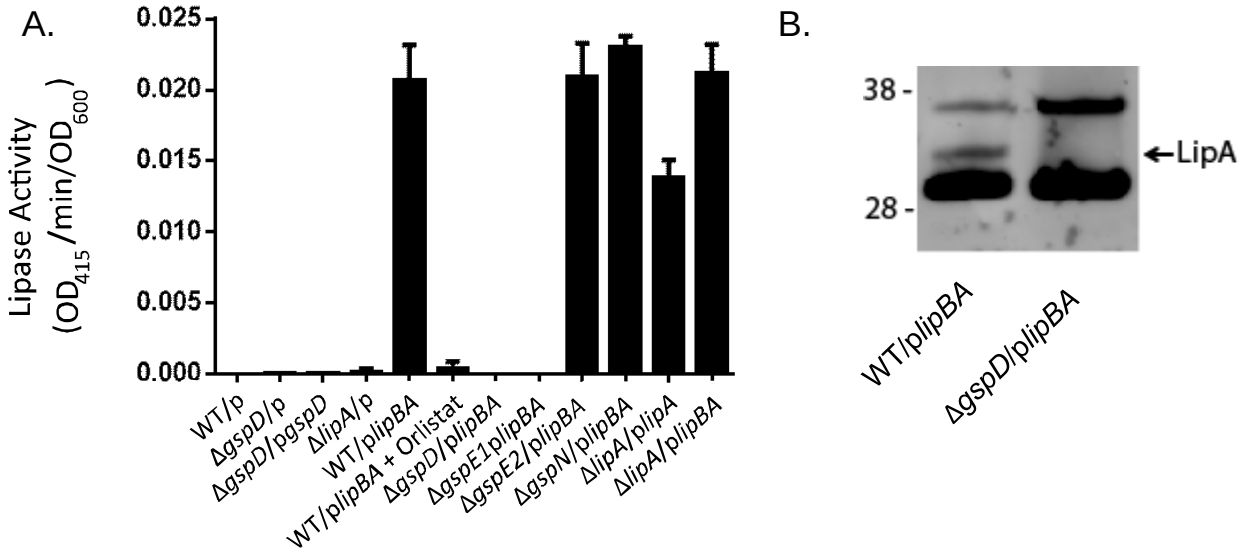


Figure 2.4. Extracellular *A. baumannii* lipase activity. A. Enzymatic activity of overnight culture supernatants against the lipase substrate 4-nitrophenol myristate was measured as a change in absorbance at 415 nm per minute and normalized to the absorbance of the culture at 600 nm. Bars show standard deviation from the mean (*p≤0.05 and **p≤0.001). B. Concentrated culture supernatants of stationary phase WT/p*lipBA* and Δ*gspD/*p*lipBA* cultures were subjected to SDS-PAGE and immunoblot analysis using LipA antibodies.

Characterization of the secreted substrate, LipA. The *A. baumannii* T2S substrate, LipA, is 54% identical to the well-characterized *P. aeruginosa* LipA, which is a member of the proteobacterial lipase homology group I (137). Lipases within this group require helper proteins (chaperones) for their proper folding (138, 139). This relationship appears to be true in *A. baumannii* as well. The Δ*lipA*/p*lipA* strain, which only overexpresses the lipase, exhibited lower extracellular lipase activity than the Δ*lipA*/p*lipBA* strain, where both the lipase and the chaperone are overexpressed (Figure

2.4). This difference is likely due to a reduction in secretion of LipA, as proper folding of LipA is required for the outer membrane translocation of LipA via the T2S system in *Pseudomonas glumae* (139). Members of the lipase homology group I are serine hydrolases with active sites containing the residues Ser-His-Asp/Glu (140, 141). Consistent with the overall high sequence homology, sequence alignments indicate that the *A. baumannii* LipA has the catalytic residues Ser-His-Asp (Figure 2.3). To further examine the catalytic property of LipA, we used the ActiveX Serine Hydrolase probe, which binds covalently to the serine nucleophile in the active site of serine hydrolases. As LipA activity is not detected in the supernatant of WT *A. baumannii,* we incubated this probe with supernatants of WT/p*lipBA* and Δ*gspD*/p*lipBA* cultures induced with increasing amounts of IPTG. In the WT/p*lipBA* samples, we observed a band increasing in intensity with increasing IPTG induction that corresponded to a protein of ~32 kDa, the expected size of LipA (Figure 2.5). In contrast, this band was not present in any of the Δ*gspD*/p*lipBA* samples. Taken together with the sequence homology between LipA of *A. baumannii* and *P. aeruginosa*, these findings indicate that *A. baumannii* LipA is a secreted serine hydrolase belonging to the lipase homology group I.

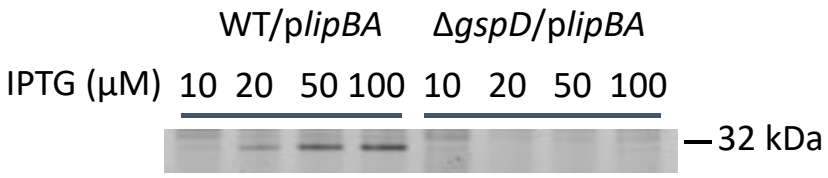


Figure 2.5. The ActiveX Serine Protease Probe binds to LipA. Culture supernatants isolated from overnight WT/plipBA and ΔgspD/plipBA cultures induced with increasing concentrations (μM) of IPTG were incubated with the ActiveX Serine Protease probe for 1 hr. Samples were subjected to SDS-PAGE and Typhoon image analysis as described in the Methods.

Orlistat is a semisynthetic, therapeutic drug prescribed for the treatment of obesity. It binds to the active site of human pancreatic lipase, thereby preventing the

enzyme from breaking down dietary lipids in the intestine (142, 143). By incubating culture supernatants of WT/p*lipBA* with Orlistat, we learned that it also inhibits *A. baumannii* LipA (Figure 2.4). Through its ability to inhibit LipA, Orlistat was therefore capable of inhibiting growth of *A. baumannii* when added to lipid agar, but not to LB agar (Figure 2.2). Similarly, when the recently isolated clinical MDR strains of *A. baumannii* AYE, 0057, and 5075 were analyzed, they were able to grow on lipid agar in the absence, but not in the presence, of Orlistat (Figure 2.2B), suggesting that these MDR isolates also secrete lipases that can break down lipids and support fatty acid metabolism.

The T2S system and its secreted substrate, LipA, are required for *A. baumannii* colonization in a murine bacteremia model. We utilized a modified murine bacteremia model originally developed for uropathogenic *E. coli* to determine if the T2S system and its substrate, LipA, are required for *A. baumannii in vivo* fitness (144). Due to the low virulence of *A. baumannii* ATCC 17978 in mice (145), we used an experimentally-induced leukopenic mouse model (146) by treating the mice with cyclophosphamide prior to infection. To diminish the potential of confounding factors between mice, we co-infected each mouse with equal numbers (1×10^7 CFUs/mL) of either WT and Δ*gspD* or WT and Δ*lipA* strains. After 24 hr, mice were euthanized and the spleens and livers were harvested and homogenized. Homogenates were plated on LB agar with and without kanamycin, CFUs were determined, and the competitive index (CI) was calculated for each mutant strain as described in Material and Methods (Figure 2.6). A CI value less than 1 indicates a colonization defect of the mutant relative to the WT strain. Both mutants were out-competed by the WT strain and exhibited colonization

defects in both the spleen and the liver. The *ΔgspD* strain had a greater disadvantage in the spleen than the liver (Spleen: CI=0.024±0.787, p-value < 0.0001; Liver: CI=0.163±0.172, p-value < 0.0001) and had a lower CI than the Δ*lipA* strain in the spleen (CI=0.096±0.260, p-value < 0.0001). The reduction in colonization was not simply due to the presence of the kanamycin resistance cassette, as a strain with the transposon Tn5 containing the kanamycin resistance gene inserted into the *ddc* gene of *A. baumannii* had no colonization defect in the spleen compared to the WT strain (146). In addition, the reduction in colonization of the Δ*lipA* and Δ*gspD* mutants is unique to the *in vivo* environment, as *in vitro* competition experiments in LB broth did not show a difference between WT and Δ*lipA* or WT and Δ*gspD* strains (CI=0.98±0.76 and CI=0.84±0.58, respectively).

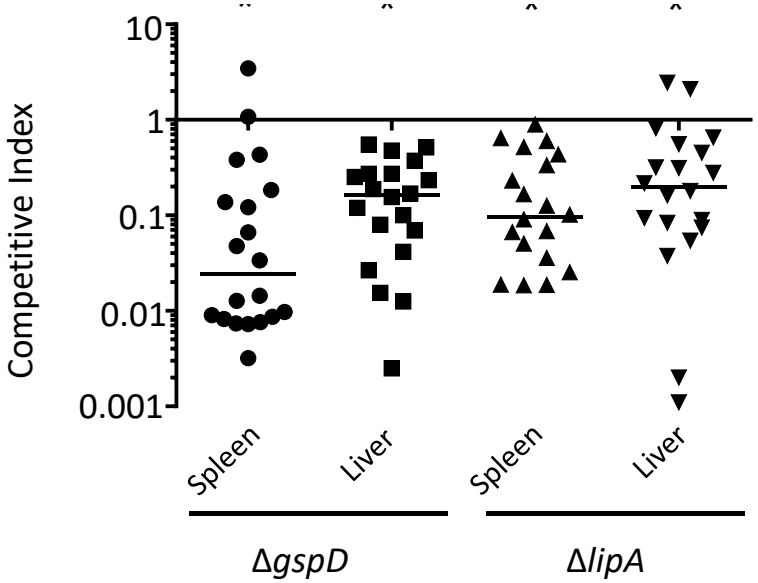


Figure 2.6. The *gspD* and *lipA* genes are required for *A. baumannii* fitness in a mouse model of bacteremia. Mice were co-inoculated with equal numbers of either WT and ΔgspD or WT and ΔlipA strains. Mice were euthanized 24 h post infection, the organs were harvested and the CFUs were determined for each strain. The competitive indices were calculated as ([mutant CFU/WT CFU]/[mutant Input CFU/WT Input CFU]). For all four competitions, p-values <0.001**.**

Discussion

The T2S system controls the secretion of toxins and hydrolytic enzymes required for virulence in a variety of pathogens. Here, we present the first study that identifies a functional T2S system in *A. baumannii*. Not only does *A. baumannii* possess all the genes for a T2S system, but there is a measurable reduction in extracellular LipA activity and loss of growth on lipid agar when *gspD* and *gspE1*, two of the core T2S genes, are deleted. In contrast, deletion of *gspE2* has no effect on lipase secretion in *A. baumannii*. While GspE1 supports T2S and the homologous ATPase A1S_0329 likely provides energy for type IV pilus assembly, it is not clear what process GspE2 participates in, if any. Perhaps it affects T2S or type IV pilus biogenesis under very specific conditions. The finding that deletion of *gspN* had no effect on lipase secretion is

consistent with its absence in many bacteria with functional T2S systems. In kind, the *gspN* homolog *pulN* is not required for pullulanase secretion via the T2S system in *Klebisella oxytoca* (147). Similarly to GspE2, GspN may support T2S only under very specific growth conditions. Alternatively, it may substitute for GspC to support secretion of T2S substrates other than LipA (see below).

As mentioned earlier, the T2S genes are distributed throughout the genome in multiple operons in *A. baumannii* rather than in one or two operons typical of other organisms encoding T2S systems. These smaller operons contain 1-4 *gsp* genes and, in some cases, may represent functional units that encode T2S components that directly interact. For example, GspC and GspD interact via the periplasmic domain of GspC, and mutations that interfere with their interaction have a negative impact on secretion (59, 60). Interestingly, *Xanthomonas campestris* does not have a *gspC* gene; instead it expresses GspN, which is required for secretion and interacts with GspD (72). Two proteins of the inner membrane platform, GspM and GspL, are encoded on another operon, and, in previous studies, we have demonstrated that these proteins stabilize each other within the cytoplasmic membrane and can be co-immunoprecipitated (57). The minor pseudopilins GspI, GspJ and GspK interact and may form a subcomplex at the tip of the T2S pseudopilus (148), and they are encoded by the same operon that also contains the minor pseudopilin *gspH* gene. The genes for the inner membrane protein GspF and the major pseudopilin GspG are located in their own operon. Currently, there is no known interaction between GspF and GspG; however, the co-localization of their respective genes suggests a potential functional interaction between these proteins in the T2S complex. Finally, while the cytoplasmic

ATPase GspE forms a stable complex with GspL at the cytoplasmic membrane (55, 56), both *gspE1* and *gspE2* are encoded on their own individual operons separate from *gspL*. The reason for this is not known, but as discussed above, may suggest that GspE1 and GspE2 may be used under different conditions or for the secretion of different substrates. The unusual arrangement of the T2S genes presents an interesting avenue for further study.

While the exact role of LipA is currently unknown, it may be required for nutrient acquisition by breaking down lipids or long chain fatty acids into shorter forms that are imported by the bacterium and used as carbon sources and energy through β-oxidation (149). Besides being consumed as nutrients, fatty acids derived through lipid hydrolysis may be used as signaling molecules. Specifically, fatty acid signals are capable of reverting persistor cells back to a metabolically active state (150). The signaling molecule *cis*-2-dodecenoic acid contributes to virulence in cystic fibrosis *Burkholderia cenocepacia* infections (151), (152). While lipases may not have been considered as virulence factors in the past, the *P. aeruginosa* lipases LipA and LipC contribute to motility, biofilm formation, pyoverdine production, and rhamnolipid production (153-155). Other lipases cleave host lipids to generate fatty acids that are integrated into the pathogens' own membranes (156). In addition, the extracellular esterase of Group A Streptococcus reduces phagocyte recruitment by hydrolyzing platelet activating factor PAF (157), and lipases produced and secreted by the opportunistic fungal pathogen *Candida albicans* support colonization and penetration of host cells (158, 159). Determining whether the *A. baumannii* LipA is involved in nutrient acquisition, signaling, or playing another important role in virulence are avenues for future work.

The remarkable ability of *A. baumannii* to develop resistance to multiple antibiotics underscores the necessity for novel treatment approaches. Therapeutics that disarm *A. baumannii* or reduce its *in vivo* fitness are promising alternatives. One possibility is to target LipA with a lipase inhibitor such as Orlistat. Alternatively, therapeutic targeting of secretion systems that transport multiple virulence factors to the exterior of the bacterium may be of particular interest, as their inactivation should have a greater impact than the targeting of individual virulence factors. It is quite possible that *A. baumannii* secretes additional proteins via the T2S besides LipA based on the following observations. First, the T2S system is active and supports secretion whether exogenous lipids are present or not, while *lipA* expression appears to require lipids/fatty acids (Figures 2.2A and 2.4). Second, the T2S is commonly required for the secretion of several proteins in other species. For example, proteomic analyses of culture supernatants of *L. pneumophila*, *V. cholerae* and *Burkholderia pseudomallei* indicate that greater than 20 different proteins may be dependent on the T2S system for extracellular release (75, 85, 160). Future work will focus on identifying additional T2S substrates and determining their role in *A. baumannii* pathogenesis.

Table 2.1. Plasmids and Bacterial Strains

Strain or Plasmid	Relevant Characteristics	Reference or Source
Plasmids		
pK18mobsacB	Suicide vector containing *sacB* (Km^r)	(161)
pCVD442	Suicide vector containing *sacB* (Ap^r)	(162)
pMMB67EH	Low-copy, IPTG-inducible vector (Ap^r)	(163)
p*gspD*	pMMB67EH-*gspD*	This Study
p*gspE1*	pMMB67EH-*gspE1*	This Study
p*gspE2*	pMMB67EH-*gspE2*	This Study
p*gspN*	pMMB67EH-*gspN*	This Study
p*lipA*	pMMB67EH-*lipA*	This Study
p*lipBA*	pMMB67EH-*lipBA*	This Study
E. coli Strains		
MC1061	F- *lac* mutant; K-12 laboratory strain	(164)
MM294/pRK2013	Helper strain for conjugation	(165)
SY327λpir	λpir lysogen; permits replication of pCVD442	(166)
A. baumannii strains		
AYE	Clinical Strain	(167)
AB0057	Clinical Strain	(168)
AB5075	Clinical Strain	(169)
17978	Wild type for T2S	ATCC
Δ*gspD*	Replacement of *gspD* with *aph-3* (Km^r)	This Study
Δ*gspE1*	Replacement of *gspE1* with *aph-3* (Km^r)	This Study
Δ*gspE2*	Replacement of *gspE2* with *aph-3* (Km^r)	This Study
Δ*gspN*	Replacement of *gspN* with *aph-3* (Km^r)	This Study
Δ*lipA*	Replacement of *lipA* with *aph-3* (Km^r)	This Study

Table 2.2. Primers used for plasmid construction

Primer	Sequence (5'-3')	Construct(s) Generated
KanUp	CCGGAATTGCCAGCTGGG	Δ*gspD*, Δ*gspE1*, Δ*gspE2*, Δ*gspN*, and Δ*lipA*
KanDown	TTCAGAAGAACTCGTCAAG	Δ*gspD*, Δ*gspE1*, Δ*gspE2*, Δ*gspN*, and Δ*lipA*
gspD1	GGTTCAACTTCTTACCAATT	Δ*gspD* vector
gspD2	CCCAGCTGGCAATTCCGGTAAAGCCATAACTCGCGA	Δ*gspD* vector
gspD3	CTTGACGAGTTCTTCTGAAGCGCCGTAGTAGCATGTTA	Δ*gspD* vector
gspD4	CGGTGCGGGTTTTGGCACAG	Δ*gspD* vector
gspD5	CGCGGATCCGTCACCATAAGAGTTAGGAA	p*gspD*
gspD6	CGCGCATGCCATAACATGCTACTACGGCGCTG	p*gspD*
gspE1-1	GTTAAACAGACTTCACGCTG	Δ*gspE1* vector
gspE1-2	CCCAGCTGGCAATTCCGGGTTTCAGG	Δ*gspE1* vector
gspE1-3	CTTGACGAGTTCTTCTGAAAGTGAAG	Δ*gspE1* vector
gspE1-4	GGTGCTGTAACTAACCCAG	Δ*gspE1* vector
gspE1-5	GACGAGCTCCTCATCATTATAAATTGT	p*gspE1*
gspE1-6	GACGTCGACGCATTTTTTATATCTTAC	p*gspE1*
gspE2-1	GAGCCTAGTCTCTTTTTTAA	Δ*gspE2* vector
gspE2-2	CTCTTGCGACATGACTTGCTTTTTCTTCATTCAGCC	Δ*gspE2* vector
gspE2-3	CTTGACGAGTTCTTCTGACCATTAAATTAAATTTTT	Δ*gspE2* vector
gspE2-4	CTACACGTTTTAAAGGCTTATAATC	Δ*gspE2* vector
gspE2-5	GACGTCGACATAGATGAGGTGAATCTT	p*gspE2*
gspE2-6	GACGAATTCATATATTGGGGAAAACAC	p*gspE2*
gspN1	GTTGAACAGCTTCTAGAATTTGGCG	Δ*gspN* vector
gspN2	CCCAGCTGGCAATTCCGGCTTTTTCTTCATTCAGCC	Δ*gspN* vector
gspN3	CTTGACGAGTTCTTCTGAGGTGGTAACTAATGAAAG	Δ*gspN* vector
gspN4	GCTCTGTAGGTTGAGACGGTGTAGC	Δ*gspN* vector
gspN5	CACGAATTCCATGTTGGTAAGGCTGAATG	p*gspN*
gspN6	GCGAAGCTTCCATACTTTCATTAGTT	p*gspN*
lipA1	AAGCTTGTCGACTTACACACACGTAC	Δ*lipA* vector
lipA2	GTTGCATGCCGGTTAAAACCCGCCAT	Δ*lipA* vector
lipA3	TTAGAGCTCCAAGGATTATAAGCTTT	Δ*lipA* vector
lipA4	TTACCCGGGTTGATATGCGCTTTA	Δ*lipA* vector
lipA5	GAGGAATTCAGTAAAAAATGAAAAGG	p*lipA*
lipA6	GAGGTCGACTAAAGCGTAAGCTTATA	p*lipA*
lipBA1	CAACGAGCTCAAACTTAAGGAAGATA	p*lipBA*
lipBA2	GAGGTCGACTAAAGCGTAAGCTTATA	p*lipBA*

Chapter III:

The Adamalysin-like Protease CpaA Secreted by *Acinetobacter baumannii* Promotes *in vivo* Colonization and Inactivation of Coagulation Factor XII

By: Ursula Waack, Mark Warnock, Zachary Huttinger, Andrew Yee, Sara Smith, Ayesh Kumar, David Ginsburg, Harry Mobley, Daniel Lawrence, and Maria Sandkvist

Abstract

Antibiotic resistant *Acinetobacter baumannii* is increasingly recognized as a cause of difficult-to-treat nosocomial infections. It generally infects immune compromised individuals, causing pneumonia, skin and wound infections, urinary tract infections, and bacteremia. Previous work has demonstrated that the metalloprotease CpaA is secreted by the Type II Secretion System (T2SS) and prolongs clotting time when added to human plasma as measured by the Activated Partial Thromboplastin Time (aPTT) assay. Here, we show that CpaA interferes with the intrinsic coagulation pathway but has no detectable effect on the extrinsic pathway. By utilizing a modified aPTT assay, we demonstrate that factor XII (fXII) is the target of CpaA. In addition, we map the cleavage site of fXII to the glycosylated proline-rich region of fXII between residues Pro308 and the O-linked glycosylated Thr309. While deglycosylation of fXII did not affect its activity, it prevented cleavage of fXII by CpaA. Deletion of the *cpaA* gene

resulted in a mutant that is outcompeted by the wild-type *A. baumannii* strain following intravenous co-inoculation of mice. As a determinant of *in vivo* fitness, CpaA may prevent activation of the intrinsic pathway by cleaving fXII, thus allowing *A. baumannii* to escape, without being walled off by clots, and disseminate.

Importance

Infections by *A. baumannii* are becoming more dire due, in part, to the bacteria's high propensity to gain antibiotic resistance. This study aimed to gain further insight into how a secreted substrate, CpaA, aids bacterial survival in the bloodstream. We verified the necessity of CpaA for the full colonization using a murine bacteremia model. In addition, we showed that CpaA cleaves a clotting component, fXII. This cleavage is dependent on the presence of glycosylation. This work also provides insight into the possible physiological role of fXII in bacterial infections.

Introduction

Antibiotic resistance in community and health care settings is increasingly common and is especially alarming for gram-negative pathogens as they are rapidly becoming resistant to the majority of available drugs (170). Among the more serious health care-associated gram-negative infections are those caused by antibiotic resistant *Acinetobacter baumannii* (http://www.cdc.gov/drugresistance). While rarely acquired in the community, the recently emerging pathogen *A. baumannii* survives remarkably well in the hospital environment and adapts easily in individuals with compromised immune defenses where it is capable of causing pneumonia, skin, wound, and urinary tract infections, bacteremia and meningitis (171). The escalating prevalence of life-

threatening infections caused by multidrug-resistant strains of *A. baumannii* highlights the urgent need for new therapeutic interventions. Analysis of mutants with single gene deletions or transposon insertions in different infection models has identified a number of *A. baumannii* factors important for *in vivo* persistence that may be suitable for therapeutic targeting (31, 32, 146, 172-174). For example, survival in the blood stream and subsequent colonization by *A. baumannii* in the spleen and liver is in part supported by the type II secretion system (T2SS), a multiprotein complex that transports a variety of enzymes, including lipases and proteases, across the Acinetobacter outer membrane (174). This was demonstrated using a neutropenic murine model for bacteremia, in which the T2SS mutant ∆*gspD* was outcompeted by the wild type (WT) *A. baumannii* strain. Similar colonization defects were observed with other *gspD* mutants of *A. baumannii* and the closely related *A. nosocomialis* in murine pulmonary infection models (175, 176). LipA, a lipase that is secreted by the T2SS and required for lipid utilization, contributes to the *in vivo* colonization of *A. baumannii* (174). However, the Δ*lipA* mutant does not show the same colonization deficiency as the Δ*gspD* mutant in the bacteremic murine model, suggesting that additional proteins delivered by the T2SS may have a positive influence on the survival of *A. baumannii* in the mammalian host.

Recently, the metalloprotease CpaA was shown to be secreted by the T2SS in *A. nosocomialis* (175). In addition to a functional T2SS, it was demonstrated that CpaA folding and/or secretion also requires CpaB, a putative chaperone. CpaA is also expressed by several *A. baumannii* isolates including AB031 and has been suggested to interfere with blood coagulation, as cell-free culture supernatant from AB031, but not that of a *cpaA* deletion mutant, increases the clotting time of normal human plasma in

an Activated Partial Thromboplastin Time (aPTT) assay (177). The aPTT assay primarily measures the activity of the contact activation and common pathways of coagulation and is routinely used in combination with the Prothrombin Time (PT) assay for the screening of coagulation factor deficiencies.

The contact activation (or intrinsic) pathway is initiated when circulating coagulation factor XII (Hageman factor; fXII) comes in contact with negatively charged artificial or biological surfaces such as polyphosphates, nucleic acids, phospholipids, misfolded proteins or lipopolysaccharides (LPS) resulting in its autoactivation (178). Active fXII (fXIIa), in turn, cleaves and activates Factor XI. In a subsequent cascade of proteolytic events, which generates thrombin and induces fibrin polymerization, a clot is ultimately formed (Figure 3.1). FXIIa also activates prekallikrein (PK) to generate kallikrein, which in reciprocal fashion produces more fXIIa through a feedback loop. In addition, kallikrein targets high-molecular-weight kininogen to release bradykinin, a short vasodilator peptide.

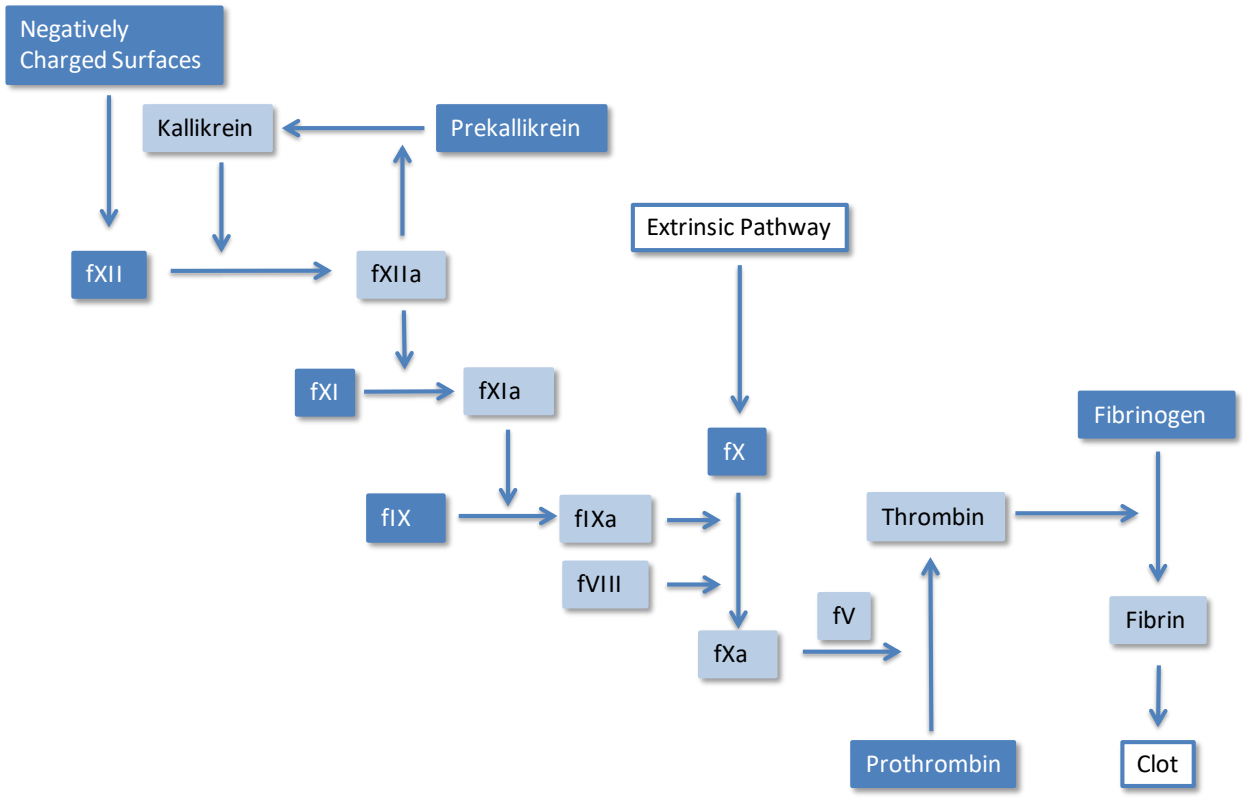


Figure 3.1. Schematic of the contact activation (intrinsic) pathway of coagulation. f, factor.

Here, we show that robust colonization of *A. baumannii* strain AB031 in a murine bacteremia model is supported by the T2SS, in part through the secretion of CpaA to the extracellular environment. We also demonstrate that CpaA increases the clotting time in the aPTT assay through proteolytic cleavage and inactivation of fXII, both in human and murine plasma. Specifically, CpaA targets the glycosylated, proline-rich domain of fXII. While deglycosylation of fXII does not interfere with its activity, it prevents cleavage by CpaA.

Materials and Methods

Bacterial Strains and Growth Conditions

Acinetobacter baumannii ATCC 17978, AB031 and their isogenic mutants were cultured in Luria-Bertani (LB) broth at 37° C. 100 µg/ml of carbenicillin or 50 µg/ml of kanamycin was used for plasmid maintenance.

Construction of Δ*gspD* strains

Chromosomal DNA isolated from the AB031 strain was used as the template for PCR. PCR reactions were carried out with Phusion DNA polymerase. Primers were synthesized by IDT Technologies.

To generate the AB031Δ*gspD* strain, we used a previously made plasmid construct pCVDΔ*gspD*(174). This construct was conjugated from the *Escherichia coli* strain SY327λpir into the AB031 *A. baumannii* strain. Transconjugates in which pCVDΔ*gspD* had recombined into the *A. baumannii* genome were selected on LB agar containing carbenicillin. To select for the second recombination event, individual colonies were cultured overnight in LB broth, diluted, cultured to late log phase, and spread on LB agar containing 3% sucrose. Sucrose and kanamycin resistant and carbenicillin sensitive isolates were considered positive for the recombination event.

Construction of p*cpaA* and p*cpaAB* plasmids

The *cpaA* and *cpaB* genes were amplified from chromosomal AB031 DNA using primers shown in Table 3.2. The 2,532 bp product was digested with SacI and SphI and

ligated into a low-copy, broad-host vector pMMB67EH to make p*cpaAB*. The construct was verified by sequencing and conjugated from the *E. coli* strain MC1061 into WT and Δ*gspD A. baumannii* 17978 strains to make WTp*cpaAB* and Δ*gspD*p*cpaAB*.

In vivo competition assay

Competition assays were carried out as described previously(174). Briefly, overnight cultures of AB031 and AB031Δ*gspD A. baumannii* strains were diluted in phosphate buffer saline (PBS). Inocula of 10^7 cells of equal amounts of AB031 and AB031Δ*gspD* were administered via tail-vein injection into eight-week old female CBA/J mice (Jackson Laboratory). Spleens and livers were removed after mice were euthanized 24 h post infection. Organs were homogenized in PBS and plated onto LB agar with and without kanamycin. CFU counts were determined and used to calculate the competitive index (CI). CI = ([mutant CFU/WT CFU]/[mutant Input CFU/WT Input CFU]). Competition assays with AB031 and AB031Δ*cpaA* were carried out in the same way.

Concentration of Supernatant

Cultures (10 mL) of each *A. baumannii* strain were grown for 16 hours in LB. The supernatant was separated from the cells by centrifugation at 1250 x g for 10 min and filter sterilized. The supernatants were concentrated 100X using Amicon Ultra Centrifugal Filter with a 30 kDa cutoff.

Activated partial thromboplastin time (aPTT) assay

Concentrated cell-free supernatants were mixed with normal human pooled plasma (George King) for 20 min at 37° C. An equal volume of aPTT reagent (Pacific Hemostasis) was added. After incubation for five minutes, $CaCl_2$ (final 10mM) was added. Clot times were determined using an Amelung KC4A Micro Coagulation Analyzer.

To identify the target of CpaA, a modified aPTT assay was performed by first incubating concentrated AB031 supernatant with normal plasma. After incubation, the plasma was diluted 1:100 and added to human plasma derived from congenital coagulation factor deficient donors (George King) and an aPTT assay was performed as described above.

Prothrombin time (PT) assay

Concentrated cell-free supernatant was incubated with normal human plasma for 20 min at 37° C. After incubation, PT reagent was added and clot times were determined using an Amelung KC4A Micro Coagulation Analyzer.

SDS-PAGE and Immunoblot Analysis

Samples were boiled in SDS sample buffer, subjected to SDS-PAGE on 4 to 12% Bis-Tris polyacrylamide gels (NuPAGE; Invitrogen), and visualized using a Typhoon Trio variable mode imager system and ImageQuant software. CpaA was

identified by liquid chromatography mass-spectrometry (LC-MS/MS) of gel-excised material (MS Bioworks, Ann Arbor, MI).

Samples were subjected to SDS-PAGE and immunoblotting using 1.25 µg/mL mouse anti-fXII biotinalyted antibodies (Molecular Innovations) for fXII, 1:5000 sheep anti-fV, and 1:5000 goat anti-fibrinogen. horse radish peroxidase (1:2000 (fXII), 1:10000 (fV), 1:1000 (fibrinogen, Thermo Scientific) secondary antibodies were used and the blot was visualized with SuperSignal West Pico Substrate (Thermo Scientific). Developed blots were imaged using a Typhoon Trio.

N-terminal Sequencing

Purified fXII was treated with CpaA-containing culture supernatants, subjected to SDS-PAGE and transferred to PVDF membrane using 10 mM CAPS, 10% methanol, pH 11. The membrane was stained with Coomassie Brilliant Blue R-250, destained, washed with water, and then air-dried. The fXII fragments were cut out and subjected to automated N-terminal sequencing (Edman degradation) using an ABI 492 protein sequencer system (Protein Chemistry Laboratory at Texas A&M University).

Deglycosylation

Purified fXII was treated with O-glycosylase (endo-α-*N*-acetylgalactosaminidase) and neuraminidase (acetyl-neuraminyl hydrolase) for 4 hr at 37° C in Glycobuffer according to the manufacturer's instructions (New England BioLabs) to remove O-linked disaccharide carbohydrates.

Statistical Analysis

Ordinary one-way Anovas were performed on the aPPT and modified aPTT tests. A Wilcoxon Signed Ranked test was used to analyze the *in vivo* murine data.

Ethics Statement

All mouse experiments were performed according to the protocol (PRO00005052) approved by the University Committee on Use and Care of Animals at the University of Michigan. This protocol is in complete compliance with the guidelines for humane use and care of laboratory animals mandated by the National Institutes of Health.

Results

We have previously shown that the T2SS supports survival of the *A. baumannii* reference strain ATCC 17978 in a bacteremia model of leukopenic CBA/J mice (174). Here, we determined whether the T2SS also promotes the *in vivo* survival of AB031, an *A. baumannii* strain that was recently isolated from the blood stream of a 55-year-old female patient (179). We substituted the *gspD* gene, which encodes the outer membrane pore of the T2SS, with a kanamycin resistance cassette and pooled the kanamycin-marked mutant cells and isogenic WT AB031 cells at a ratio of 1:1. The mice were inoculated with 10^7 colony forming units (CFU) of the bacterial pool intravenously via the tail vein. Twenty-four hr later, the mice were euthanized, and spleens and livers were harvested, homogenized and plated. In contrast to ATCC 17978 (146), AB031 did not require immune suppressed mice for infection, and inoculation of 10^7 bacteria

resulted in 10^5 CFU/liver/mouse 24 h post infection. The CFU of kanamycin sensitive (WT AB031) and resistant (Δ*gspD* mutant) bacteria were counted, and competitive indices (CI) were calculated. The mutant was outcompeted by the WT strain in both spleen and liver, indicating that the T2SS contributes to *in vivo* fitness of AB031 as well (Figure 3.2). Next we evaluated the role of the T2SS substrate CpaA in *in vivo* survival by analyzing the colonization of a Δ*cpaA* deletion strain that is deficient in the metalloprotease CpaA (177). This protease was recently identified in the T2SS proteome of *A. nosocomialis* (175), and we have confirmed that CpaA is also secreted by the T2SS in *A. baumannii* by SDS-PAGE and mass spectrometry analysis (Figure 3.3). The Δ*cpaA* deletion mutant was outcompeted by the WT strain (Figure 3.2), suggesting that CpaA is expressed and secreted *in vivo* and that it supports the survival of *A. baumannii* AB031 in the murine host.

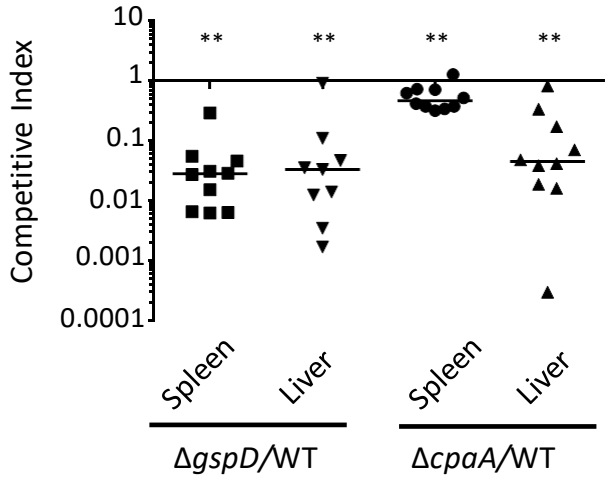


Figure 3.2. The T2SS and CpaA enhance colonization in a murine bacteremia model. Mice were co-inoculated with equal amounts of either *A. baumannii* AB031 and AB031 Δ*gspD* or AB031 and AB031 Δ*cpaA*. After 24 h, the spleen and livers were harvested and CFUs determined. The competitive index was calculated as follows: [output mutant CFU/output WT CFU]/[input mutant CFU/input WT CFU]. Only nine data points are shown for the AB031 Δ*gspD*/WT AB031 liver samples due to one mouse sample having too many CFUs to count. **p ≤ 0.01 by Wilcoxon Signed Rank Test.

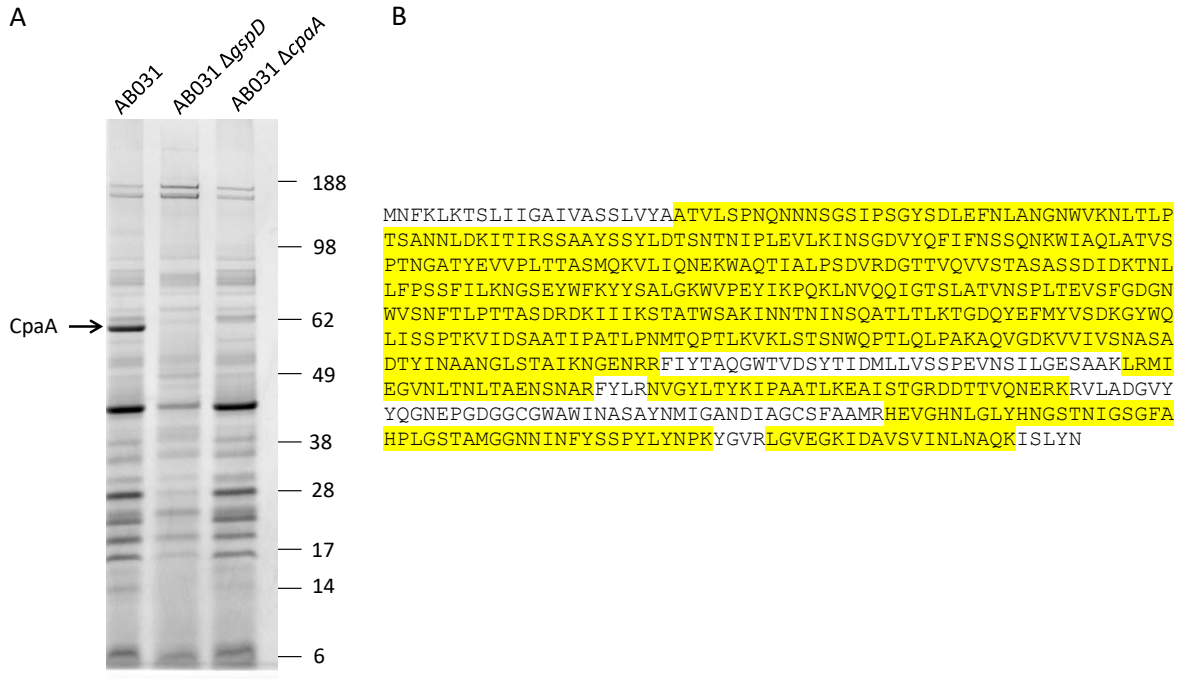


Figure 3.3. Secretion of CpaA is dependent on the *A. baumannii* T2SS. **A.** Concentrated culture supernatants from AB031 and the T2SS mutant Δ*gspD* were analyzed by SDS-PAGE and Coomassie staining. The AB031 Δ*cpaA* mutant served as a negative control. **B.** CpaA was excised from the gel and subjected to LC-MS/MS analysis, which identified 45 exclusive unique peptides, 121 exclusive unique spectra and a total of 448 spectra. The resulting sequence coverage (highlighted in yellow) was 84%. No peptide representing the signal peptide was identified as the signal peptide is removed during transport of CpaA across the cytoplasmic membrane.

CpaA, a member of the adamalysin-family of secreted metalloproteases, has been found to inhibit clotting in human plasma using the aPTT assay(177). We have followed up on these findings and confirmed that culture supernatant from the WT AB031 strain, but not from the isogenic Δ*cpaA* mutant, increases the clotting time in an aPTT assay in human plasma (Figure 3.4A). In addition, we found that CpaA produced by the WT AB031 strain also interferes with clotting of murine plasma (Figure 3.4B). Similarly to the Δ*cpaA* mutant, supernatant from the T2SS mutant Δ*gspD* had no effect on clotting (Figure 3.4). Cloning and over-expression of *cpaA* and the putative chaperone-encoding *cpaB* also resulted in extracellular release of CpaA in a T2SS dependent fashion in the reference strain ATCC 17978, which does not encode its own

cpaA and *cpaB* genes (Figure 3.5). Not surprisingly, culture supernatant from WT ATCC 17978 over expressing the protease also extended the clotting time in the aPTT assay, while expression of plasmid-encoded *cpaA* and *cpaB* in the 17978Δ*gspD* mutant did not diminish the clotting function (Figure 3.6A). The effect on the clotting time in the aPTT assay was dose dependent (Figure 3.6B), and the CpaA activity was significantly higher in the culture supernatant of ATCC 17978 than in the original AB031 due to overexpression, thus generating sufficient amounts of high activity material for subsequent analysis. While we confirmed that CpaA does inhibit clotting in the aPTT assay, unlike Tilley *et al*. (177), we found no significant effect of CpaA in a PT assay (Figure 3.7A), which measures the activity of the extrinsic pathway (177). Despite not having an effect in the PT assay, CpaA does cleave coagulation factor V (fV; Figure 3.7B), but this cleavage neither inactivates nor activates fV. These findings lead us to focus on the intrinsic pathway and the aPTT assay.

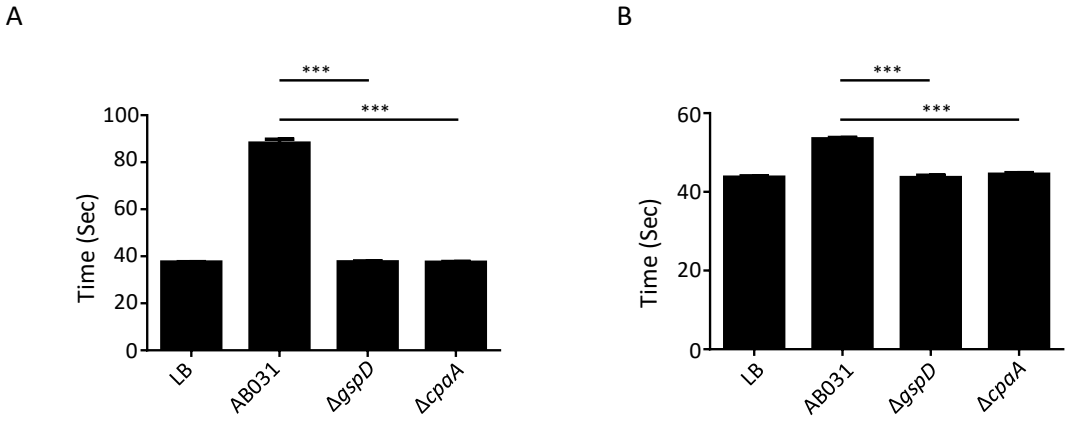


Figure 3.4. Clotting time in human and murine plasma is increased when incubated with culture supernatant from AB031 but not from strains deficient in either CpaA or the T2SS. A. aPTT after incubation of normal human plasma with culture supernatant from AB031, AB031 Δ*cpaA* or AB031 Δ*gspD*. The growth medium LB served as negative control. ***p ≤ 0.001, by ordinary one-way Anova; n=6. **B.** aPTT after incubation of murine plasma with culture supernatants from the same strains as in A or LB. ***p ≤ 0.001, by ordinary one-way Anova; n=6.

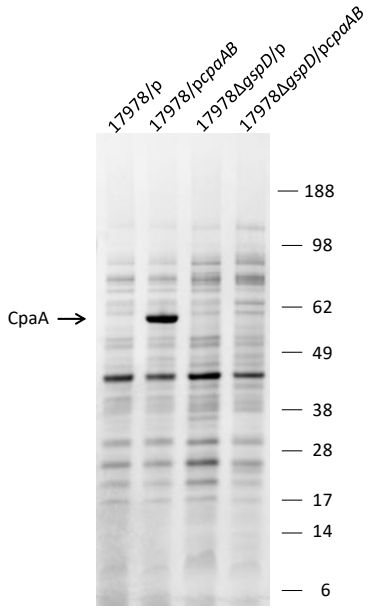


Figure 3.5. Overexpression and secretion of CpaA in the ATCC 17978 strain. Concentrated culture supernatants from ATCC17978 or the Δ*gspD* mutant containing empty vector (p) or a plasmid carrying the *cpaA* and *cpaB* genes were analyzed by SDS-PAGE and Coomassie staining.

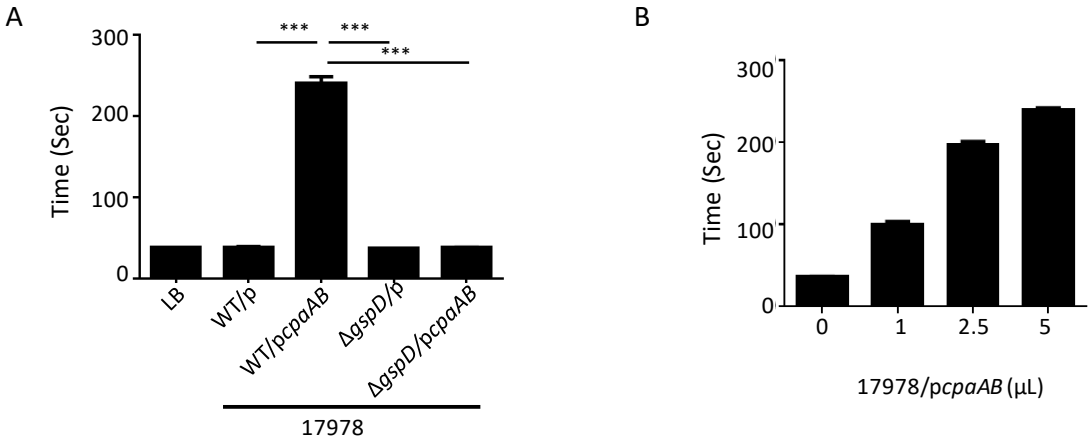


Figure 3.6. Culture supernatant from *A. baumannii* 17978 overexpressing CpaA and the putative chaperone CpaB increases the clotting time in the aPTT assay. A. aPTT assay after incubation of normal human plasma with culture supernatant from 17978 or the T2SS mutant 17978Δ*gspD* containing either empty vector (p) or p*cpaAB*. LB medium is included as a negative control. ***p ≤ 0.001, by ordinary one-way Anova; n=6. **B.** Increasing amounts of culture supernatant from 17978/p*cpaAB* were added to human plasma, and an aPTT assay was performed; n=6.

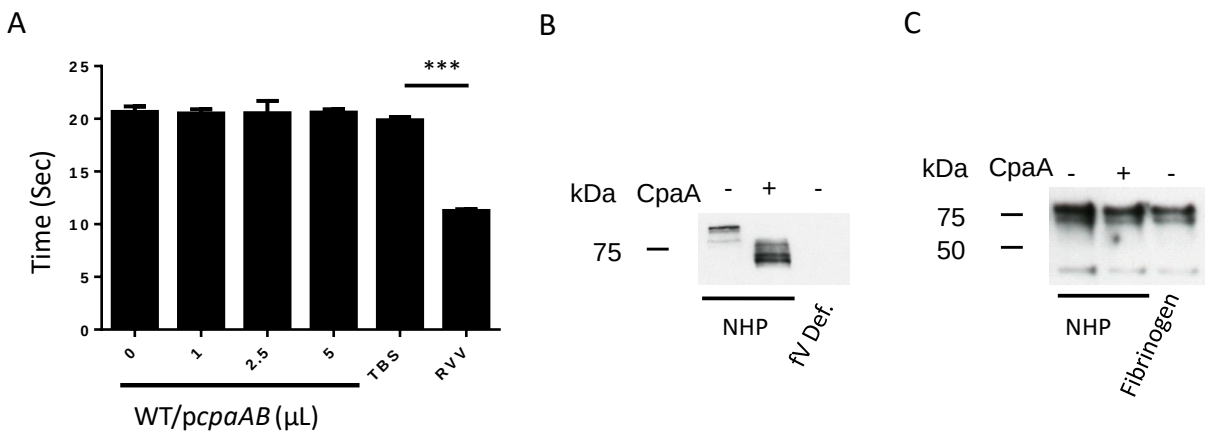


Figure 3.7. CpaA has no effect on clotting in a PT assay. A. Increasing amounts of supernatant from ATCC 17978/p*cpaAB* containing CpaA was added to normal human plasma (NHP) and analyzed for clotting using a PT assay; n=6, ***p<0.001. The snake venom protease RVV-V, a factor V activator, and the buffer TBS were included as controls. **B.** Human plasma was incubated with or without CpaA and subjected to SDS-PAGE and immunoblotting using α-fV antibody. fV deficient plasma incubated in the absence of CpaA was used as a negative control. **C.** Human plasma was incubated with or without CpaA, subjected to SDS-PAGE, and immunoblotted using α-fibrinogen antibody. Purified fibrinogen was used as control.

Since the aPTT assay primarily measures the intrinsic coagulation pathway, to identify the target of CpaA we used a modified aPTT factor assay with a variety of factor-deficient human plasmas. Normal plasma was first incubated with CpaA from culture supernatant of ATCC 17978/p*cpaAB* or LB media as control. The samples were then diluted 1:100 into various human plasmas lacking specific factors of the intrinsic pathway, and an aPTT assay performed. In this way, the factor inactivated by CpaA in the normal plasma will be unable to complement the specific factor deficient plasma. The finding that the clotting time was increased only when CpaA treated normal plasma was added to fXII deficient plasma suggested that fXII is inactivated by CpaA (Figure 3.8A). This result was confirmed by immunoblotting (Figue 3.8B), which showed that CpaA efficiently cleaves both purified fXII and fXII in human plasma. A similar factor assay was also performed with CpaA treated normal mouse plasma used to reconstitute

the factor XII deficient human plasma, confirming that fXII is the target of CpaA in both human and mouse plasma (Figure 3.8C).

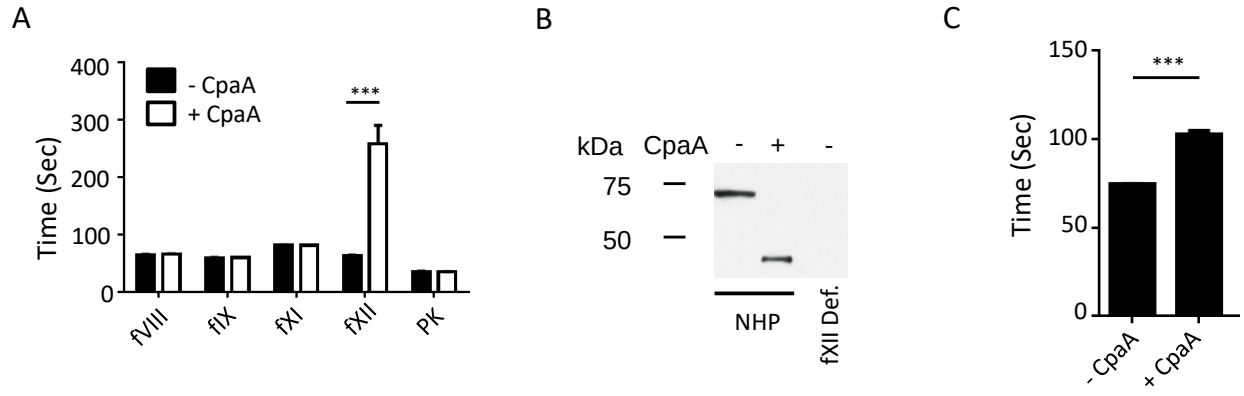


Figure 3.8. CpaA cleaves fXII. A. Normal human plasma was incubated with (+ CpaA) or without (- CpaA) culture supernatant from 17978/p*cpaAB*, diluted 1:100 and added to different factor deficient plasma as indicated. Clotting time was determined using an aPTT assay. ***p ≤ 0.0001, by ordinary one-way anova; n=6. **B.** Normal human plasma (NHP) was incubated with (+) or without (-) CpaA present in culture supernatant from 17978/p*cpaAB* for 20 min. fXII deficient control incubated without CpaA was used as a control. fXII was detected using α-fXII antibody. **C.** Murine plasma incubated with or without CpaA, diluted 1:100, added to fXII deficient plasma and tested in an aPTT assay. ***p ≤ 0.001, by ordinary one-way Anova; n=6.

SDS-PAGE and coomassie staining indicated that treatment of purified human fXII with CpaA resulted in 3 distinct bands (Figure 3.9A; lane 3). The bands were excised from the gel, and the cleavage site(s) was identified by N-terminal protein sequencing via Edman degradation. The single low molecular weight band (filled arrow) represents the N-terminal fXII fragment (not shown). The two slower migrating fragments (lane 3; open arrows) possessed identical N-termini, XXRTPPQSQX, indicating that they are the same species with possible differences in glycosylation or C-terminal heterogeneity (Figure 3.9B). The first two residues and the last marked as X could not be identified by N-terminal sequencing as they are O-linked glycosylated threonines [SwissProt entry P00748 and (180)]. However, as residues 3 through 9 were identified for both fragments, the results suggest that the sequence represents residues 309-318 in the proline-rich domain of fXII and that fXII is cleaved between Pro308 and

Thr309. Interestingly, Thr309 is modified with an O-linked mucin-type HexHexNAcNeuNAc glycan (109), suggesting that CpaA may be specific for O-linked glycosylated residues. To determine whether glycosylation is required for CpaA cleavage, we treated fXII with O-glycosidase and neuraminidase to deglycosylate fXII before incubating it with CpaA. SDS-PAGE analysis indicated that deglycosylation resulted in increased mobility of fXII (Figure 3.9A; lane 4), but more importantly, prevented cleavage by CpaA (Figure 3.9A; lane 5). The effect of deglycosylated fXII with and without treatment with CpaA was also analyzed in a modified aPTT assay in which we determined the ability of purified fXII to restore normal clotting to fXII deficient plasma. The results in Figure 3.9C show that deglycosylated fXII fully restored clotting when added to fXII deficient plasma, whether it had been incubated with CpaA or not, providing further support that deglycosylated fXII is resistant to CpaA.

It is possible that fXII is not the only physiological target of CpaA. Even though we did not observe an effect in clot formation, fV is also cleaved by CpaA (Figure 3.7). It is possible that cleavage of fV does not interfere with activation of fV into fVa. Perhaps there is a common O-linked glycosylation recognition site in fXII and fV, but only cleavage of fXII leads to inactivation. Tilley *et al.* also suggested that fibrinogen may be a target of CpaA as well(177), but we were unable to detect cleavage of fibrinogen in plasma (Figure 3.7).

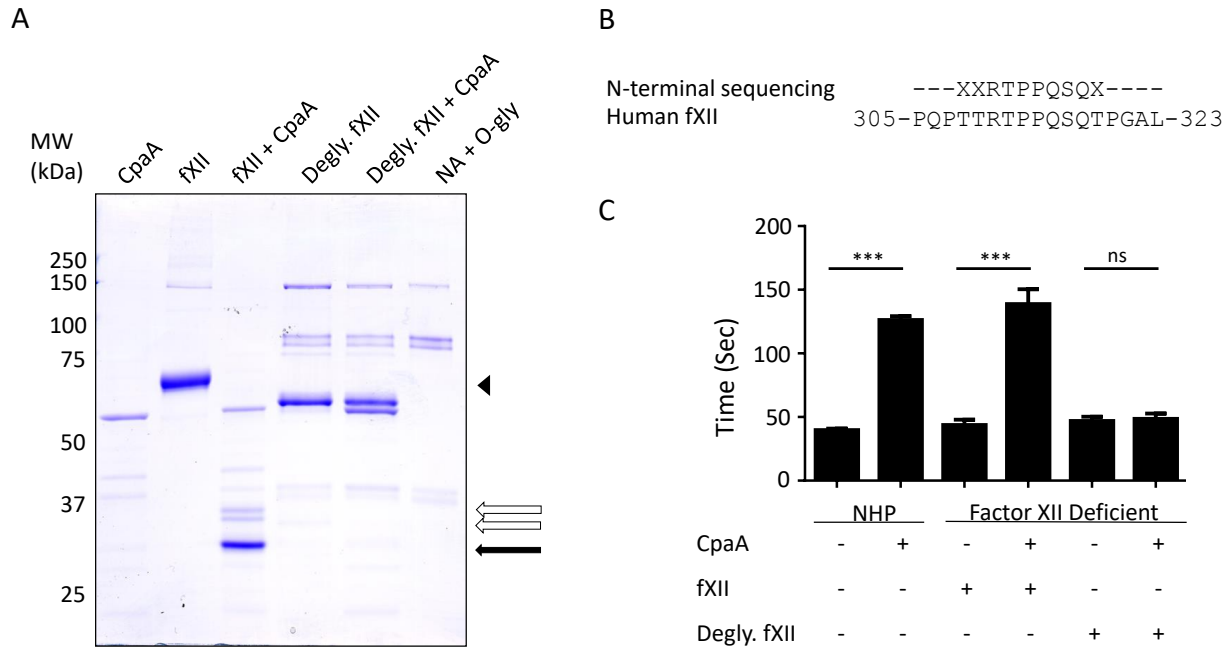


Figure 3.9. Glycosylation is necessary for cleavage of fXII by CpaA. A. Purified fXII was incubated without and with neuraminidase and O-glycosidase and then subjected to culture supernatant containing CpaA followed by SDS-PAGE and comassie staining. Neuraminidase (NA) and O-glycosidase (O-gly) are shown as controls. The positions of CpaA (arrow head) and cleaved fXII fragments (open and filled arrows) are indicated on the right. **B.** Alignment of N-terminal sequencing result of the fXII fragments (indicated by open arrows in A) with amino acid sequence of human fXII. Residues for positions 1,2 and 10 could not be identified and are marked with X. **C.** Purified fXII or deglycosylated fXII was added to fXII deficient plasma following incubation with and without CpaA and tested for clotting in the aPTT assay. Results with normal human plasma (NHP) are shown for comparison. ***p ≤ 0.001, by ordinary one-way Anova; n=6.

Discussion

Our studies have focused on the putative function of the metalloprotease, CpaA, which is secreted by the T2SS in *A. baumannii*. Both the T2SS and CpaA were shown to be required for efficient colonization of *A. baumannii* in a conventional murine bacteremia model. In addition, the clotting factor fXII was recognized as the substrate for CpaA in both human and murine plasma. N-terminal sequencing of fXII fragments, generated following treatment with CpaA, identified the cleavage site between Pro308 and the O-linked glycosylated Thr309 located in the proline-rich domain of fXII.

Glycosylation of T309 may contribute to specificity of CpaA, as deglycosylation of fXII prevents cleavage while it does not affect fXII's clotting activity *per se*.

While our previous work demonstrated the advantage of possessing the T2S system for colonization of ATCC 17978 (174), this is also true for the AB031 strain. However, a neutropenic state was not required to observe a detectable bacterial burden in the liver and spleen for AB031, suggesting that AB031 is better able to resist host defense mechanisms. The necessity of the T2S system in multiple strains of *A. baumannii* is indicative of a wide-spread role of the system and its substrates in the species as a whole. While the defect in colonization was equivalent for the spleen and the liver for the T2S mutant, there was a difference between the organs for the Δ*cpaA* mutant with the liver showing a greater difference than the spleen. While this requires further investigation, the liver is the site of fXII synthesis (181), and it is possible that *A. baumannii* is exposed to higher levels of fXII in this organ thus decreasing the competitiveness of the Δ*cpaA* mutant.

The original study that discovered fXII noted that Mr. Hageman, the individual who lacked fXII, exhibited no blood or clotting disorder(90). However, recent studies have demonstrated that fXII promotes intravascular thrombus formation (102, 182, 183). CpaA may cleave fXII to prevent the formation of clots. Without the hindrance of a clot, *A. baumannii* would be more likely to escape and gain access to other organs. Another example of a bacterium that is hypothesized to escape clot-mediated trapping is group A streptococcus (GAS). GAS produces streptokinase, which can dissolve a clot trapping

the bacterium by activating plasminogen(93). Thus, GAS destroys the clot whereas *A. baumannii* may prevent the formation of a clot.

It is possible that targeting fXII to prevent clot formation might not be the mechanism by which CpaA supports colonization. Instead or in addition, CpaA may prevent activation of the kallikrein/kinin pathway. As mentioned previously, fXIIa mediated activation of prekallikrein releases kallikrein, which, in turn, liberates bradykinin from high molecular weight kininogen (Figure 3.1). Bradykinin is a peptide hormone with a wide array of roles. Bradykinin can allow for the efflux of immune cells by increasing vascular permeability and vasodilation by binding to B-2 receptors(184). Bradykinin can be metabolized and, once metabolized, binds B-1 receptors(184). Binding of both the B-2 and B-1 receptor lead to release of immune and inflammation mediators that are immune cell dependent(185). By inactivating fXII and thus reducing the generation of bradykinin, CpaA could, in part, prevent the recruitment of immune mediators, including neutrophils and thus support survival of *A. baumannii*.

Interestingly, several mutations in the region of the CpaA cleavage site in fXII have been identified in patients with hereditary angioedema type III including two missense mutations at Thr309(107, 108). Inheritance of either Lys or Arg substitution at Thr309 (thus removing the O-linked glycosylation site) is associated with an autosomal dominant form of this disorder in multiple families. Intriguingly, these substitutions were recently shown to lead to increased contact mediated autoactivation of fXII (109). Taken together with our data, these findings raise the possibility that resistance of these mutant forms of fXII to inactivation by CpaA or other related microbial protease(s) could

provide a selective advantage to infection by *A. baumannii* balancing the manifestations of angioedema.

Table 3.1. Strains and plasmids used in this study.

Strain or Plasmid	**Relevant Characteristics**	**Reference or Source**
Plasmids		
pK18mobsacB	Suicide vector containing *sacB* (Km^r)	(161)
pCVD442	Suicide vector containing *sacB* (Ap^r)	(162)
pMMB67EH	Low-copy, IPTG-inducible vector (Ap^r)	(163)
p*gspD*	pMMB67EH-*gspD*	(174)
p*cpaA*	pMMB67EH-*cpaA*	This study
p*cpaAB*	pMMB67EH-*cpaAB*	This study
p*cpaA-kan*	pMMB960-*cpaA*	This study
p*cpaAB-kan*	pMMB960-*cpaAB*	This study
E. coli Strains		
MC1061	F^- *lac* mutant; K-12 laboratory strain	(164)
MM294/pRK2013	Helper strain for conjugation	(165)
SY327λpir	λpir lysogen; permits replication of pCVD442	(166)
A. baumannii strains		
AB031	Clinical Strain	(177)
AB031Δ*cpaA*	Replacement of *cpaA* with *aacC1* (Gm^r)	(177)
AB031Δ*gspD*	Replacement of *gspD* with *aph-3* (Km^r)	This study
17978	Wild type for T2S	ATCC
17978Δ*gspD*	Replacement of *gspD* with *aph-3* (Km^r)	(174)

Table 3.2. Primers used for plasmid construction.

Primer	Sequence (5'-3')	Construct(s) Generated
CpaA For	GAGGAGCTCTGGTTTGCTAACCTGC	p*cpaA*, p*cpaAB*
CpaA Rev	GAGGCATGC CCTATTAACAATTTAT	p*cpaA*
CpaB Rev	GAGGCATGC TCTCTACCAGAACCGTT	p*cpaAB*

Chapter IV:

Targeting the Type II Secretion System: The Development, Optimization, and Validation of a High-Throughput Screen to Identify Small Molecule Inhibitors

By: Ursula Waack, Tanya L. Johnson, Khalil Chedid, Chuanwu Xi, Lyle A. Simmons, Harry L.T. Mobley, and Maria Sandkvist

Abstract

Multi- and pan-drug resistant bacterial strains, especially nosocomial pathogens, present an increasing problem for healthcare facilities. Due to its rapid rise in antibiotic resistance, *Acinetobacter baumannii* is one of the most concerning Gram-negative species. *A. baumannii* typically infects immune compromised individuals resulting in a variety of outcomes, including pneumonia and bacteremia. Using a murine model for bacteremia, we have previously shown that the type II secretion system (T2SS) contributes to *in vivo* fitness of *A. baumannii.* Here, we provide support for a role of the T2SS in protecting *A. baumannii* from human complement as deletion of the T2SS gene, *gspD*, resulted in a 100-fold reduction in surviving cells when incubated with human serum. This effect was abrogated in the presence of serum deficient in Factor B, a component of the alternative pathway of complement activation, indicating that the T2SS protects *A. baumannii* against the alternative complement pathway. Because inactivation of the T2SS results in loss of secretion of multiple enzymes, reduced *in vivo*

fitness, and increased sensitivity to human complement, the T2SS may be a suitable target for therapeutic intervention. Accordingly, we developed and optimized a high-throughput screening (HTS) assay based on extracellular lipase activity to identify small molecule inhibitors of the T2SS. We tested the reproducibility of our assay using a 6400-compound library. With small variation within controls and a dynamic range between positive and negative controls, our assay had a z-factor of 0.65, establishing its suitability for HTS. Our screen identified every lipase inhibitor in the library demonstrating the specificity of the assay. To screen out false positives, we also developed and optimized two counter assays to eliminate inhibitors of lipase activity and lipase expression. By implementing the counter assays, we found that all seven tricyclic antidepressants present in the library are inhibitors of the lipase, highlighting the potential of identifying alternative targets for approved pharmaceuticals. Taken together, our proof-of-concept pilot study indicates that the HTS regimen is simple, reproducible, robust, and specific and that it can be used to screen larger libraries for the identification of T2SS inhibitors that may be developed into novel *A. baumannii* therapeutics.

Introduction

A growing concern in hospitals, nursing homes and other healthcare facilities is the increasing frequency of antibiotic resistant infections that result in longer hospital stays, higher costs and increased mortality. The ESKAPE pathogens *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species have attracted considerable attention as they cause the majority of nosocomial infections (11). Infections caused by *A. baumannii* are prevalent with approximately 45,000 cases per year in the United

States alone. Globally, there are about 1 million cases annually (186), and reports suggest that *A. baumannii* may be the leading cause of nosocomial infections in some countries (187). Some 50% of these infections are caused by antibiotic-resistant strains (186). Exposure to *A. baumannii* can result in a variety of infections including pneumonia, urinary tract infection, bacteremia, meningitis, skin and wound infections that may lead to sepsis (6, 9). Considered an opportunist, *A. baumannii* typically infects immune-compromised individuals, but more recently isolated strains may not be restricted to this patient population, possibly as a consequence of increased virulence (169). The remarkable ability of *A. baumannii* to form biofilm and resist dry environments (112, 118) may explain its prevalence in healthcare environments (10, 114). Additional contributing factors include multi- or pan-antibiotic resistance (6, 13), which is due, in part, to intrinsic properties of the outer membrane of *A. baumannii* and its notable ability to acquire foreign DNA through horizontal gene transfer (12).

The rise in antibiotic resistance rapidly reduces the options of effective treatment and calls for the identification of new therapeutic approaches. A recommended strategy combines antibiotics with drugs that target resistance mechanisms such as Augmentin, which consists of Amoxicillin and the β-lactamase inhibitor Clavulanate. Other feasible options include the combination of antibiotics with inhibitors of drug efflux pumps or outer membrane permeabilizers (188). Identification of new therapeutic targets is also necessary. Candidates may include essential processes such as lipopolysaccharide synthesis and transport as well as factors that contribute to *in vivo* fitness and virulence.

One of the first studies to target virulence factors using HTS of small molecule libraries identified a compound that inhibits dimerization of ToxT, a virulence regulator in

Vibrio cholerae (189, 190). This inhibitor abolishes the production of cholera toxin and decreases TCP-mediated colonization in an infant mouse model (189). Other studies have screened for biologicals or chemical compounds that target colonization factors such as curli and type 1 pili, toxins, protein secretion pathways or quorum sensing systems (188, 191-193). With a few exceptions, it is too soon to evaluate the outcome of these studies and their success; however, some of these potential anti-virulence drugs are in various stages of development and are being analyzed in animal models or clinical trials (193-195). An IgG antibody that targets the binding of anthrax toxin to its receptor is currently used as an antitoxin in combination therapy for the treatment of *Bacillus anthracis* infections (196) and demonstrates the feasibility of targeting disease-causing components of pathogens.

Secretion systems are particularly attractive targets for alternative therapeutics, as their inactivation interferes with the delivery of entire batteries of secreted virulence factors. Therefore, several HTSs have been designed to identify small molecule inhibitors of the type III secretion system, which is present in many Gram-negative human pathogens and secretes a wide variety of virulence effectors (197, 198). Another secretion system, the type II secretion system (T2SS), is responsible for the secretion of numerous degradative enzymes and toxins that contribute to survival in the environment and the mammalian host and may also be a suitable target for alternative therapeutics (48, 50, 199). As with many gram-negative pathogens, *A. baumannii* possesses a functional T2SS (174, 176). The T2SS forms an apparatus that spans both the inner and outer membrane and is encoded by 12 essential genes, *gspC-M* and *pilD* (68, 132). T2S substrates are synthesized with an N-terminal signal peptide that directs

translocation from the cytoplasm to the periplasm via the general export (Sec) or twin arginine translocation (Tat) pathways. Once in the periplasm, the signal sequence is cleaved, the protein folds, and then interacts with the T2SS to finally exit the cell via a gated outer membrane pore formed by GspD (64, 200). GspD connects to GspC, one of the components of the inner membrane platform that also consists of the transmembrane proteins GpsF, L, and M (53, 57, 59, 201). The pseudopilins GspG, H, I, J, and K make a pseudopilus, a structure homologous to the Type IV pilus, whereas PilD cleaves and methylates the pseudopilin subunits prior to their assembly (65-67). The entire system is powered by a cytoplasmic ATPase, GspE (54, 202). All of these proteins, including their expression and interactions, are potential targets for a therapeutic compound (Figure 4.1).

Recent work by our laboratory and others has demonstrated that a functional T2SS aids in *A*. *baumannii* and *A. nosocomialis* colonization (174-176). Inactivation of the T2SS or one of its substrates diminishes survival in murine models for bacteremia and pneumonia, indicating that screening for compounds that target the T2SS may identify *A. baumannii* virulence inhibitors. In this study, we describe the development and optimization of a high-throughput screen to identify small molecule inhibitors of the T2SS in *A. baumannii*. In addition, we highlight the need for inclusion of high-throughput counter-screens to remove compounds with alternative targets.

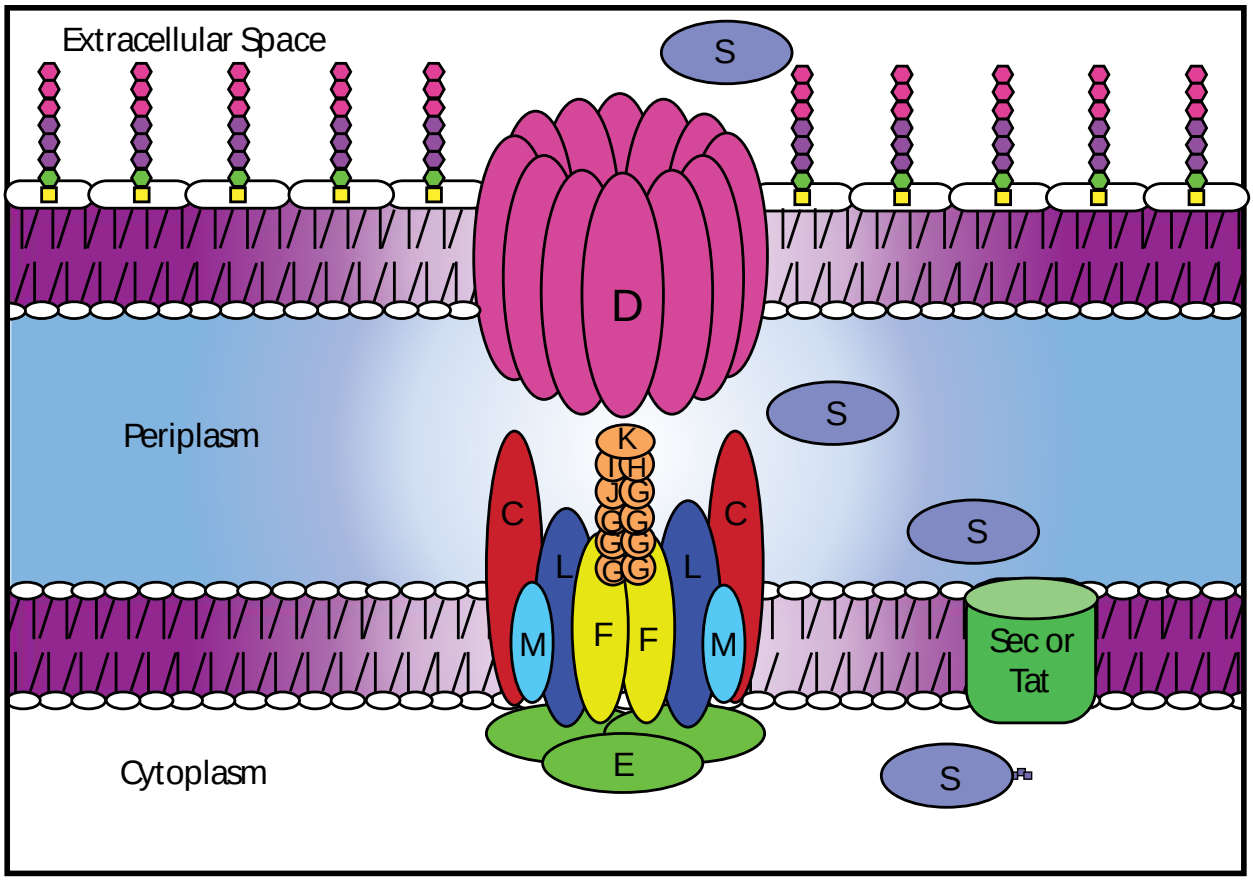


Figure 4.1. Potential Targets of a T2SS Inhibitor. A T2S inhibitor may block transcription of the T2S genes or translation, post-translational modification, protein-protein interactions or function of the T2S proteins. Blocking the recognition of the proteins to be secreted by the T2S system could also halt secretion.

Methods

Bacterial Strains and Plasmids

All bacterial strains and plasmids are described in Table 4.3. All strains were cultured overnight in Luria-Bertani (LB) broth. When necessary, LB broth was supplemented with carbenicillin (100 µg/ml) to maintain the plasmid.

Compound Library

All compounds tested in this study are from commercially available libraries acquired and maintained in 384-well plates in DMSO at -20° C at a concentration of 2

mM by the Center for Chemical Genomics at the University of Michigan. The five libraries include MS2400, NCC, Pathways, Prestwick, and LOPAC. MS2400 is a collection of FDA approved drugs plus compounds with known biological activity obtained from Microscource Discovery (Spectrum Collection). NCC is a library with compounds that have been used in human clinical trials. The Pathways collection is comprised of known active compounds with a variety of targets. The Prestwick library is composed of approved drugs which are safe for use in humans. Finally, the LOPAC collection is the Library of Pharmacologically Active Compounds from Sigma.

Serum bactericidal assay

After cultures were grown overnight in LB, the cells were separated from supernatant by centrifugation for 10 min at 3,500 rpm. The cells were washed in PBS and diluted 1:100. Equal volumes of cells and either 100% normal human serum, heat-inactivated human serum or factor depleted sera were incubated together for 30 min at 37° C. Samples were diluted and plated on LB agar to obtain CFUs.

High-Throughput Lipase Assay

Overnight cultures of wild-type (WT) *A. baumannii* 17978/p*lipBA* and 17978Δ*gspD*/p*lipBA* were grown in LB broth. After growth, the cultures were centrifuged for 10 min at 3500 rpm to separate cells and supernatant. The supernatant was removed and the pellet was washed in Mueller-Hinton 2 (MH) and resuspended in the original volume. 10 µL of MH was added to each well of a 384-well Greiner 784080 plate. Compounds in DMSO or the DMSO control were added to the wells (0.05 µL) using Perkin Elmer Sciclone liquid handler with a 50 nl pintool attachment (in primary

assay). For concentration-response assays, the TTP LabTech Mosquito X1 was used to place variable volumes (0.02 – 1.2 µL) of compounds to the wells. 17978/p*lipBA* was diluted and added to all the negative control wells as well as to the sample wells while 17978Δ*gspD*/p*lipBA* was diluted and added to the positive control wells. All cultures had a starting OD_{600} of 0.005 and were supplemented with 50 µM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce expression of the *lipBA* genes. Plates were centrifuged 1 min at 1,000 rpm to ensure all liquid was at the bottom of the well. Plates were incubated overnight at 24°C in a humidified incubator. After 16 hour incubation, the OD_{600} value of each culture was recorded. An optimized lipase assay was used to measure lipase activity. Briefly, the cultures were incubated with 0.45 mM *p*-nitrophenyl myristate, 80 mM Tris-HCl (pH 8.0), and 0.15% Triton X-100 and the absorbance at 415 nm was measured over time using the PE EnVision Multimode Plate Reader. All data were uploaded to MScreen for analysis. MScreen is a data analysis and storage system created by the Center for Chemical Genomics intended for the processing of high-throughput data generated by users of the center(203).

Lipase Inhibitor Assay

Cultures of *A. baumannii* 17978/p*lipBA* and 17978Δ*gspD*/p*lipBA* were grown overnight in LB broth with 50 µM IPTG and the supernatant and cells were separated by centrifugation. For concentration-response assays, the TTP LabTech Mosquito X1 was used to add variable volumes (0.02 – 1.2 µl) of compounds. Supernatant was added to the wells, buffer with lipase substrate was added and the change in OD_{415} was recorded as above. All data were uploaded to MScreen for analysis.

GFP Expression Assay

A. baumannii 17978/p and 17978/p*gfp* cultures were grown O/N as above. Cells were washed as described above for the lipase assay. Compounds in DMSO or the DMSO control were added to the wells of a black low-volume Greiner 784073 plate using Perkin Elmer Sciclone liquid handler with a 50 nl pintool attachment calibrated to deliver 200nM. For concentration-response assays, the TTP LabTech Mosquito X1 was used to place variable volumes (0.02 – 1.2 ul). Cultures were diluted to OD_{600}=0.005 in LB supplemented with 75 µM IPTG. Plates were centrifuged for 1 min at 1,000 rpm to ensure all liquid was at the bottom of the well and incubated overnight at room temperature in a humidified incubator. Fluorescence was measured after growth using a BMGLabtech PHERAstar (485 nm excitation, 520 nm emission). All data were uploaded to MScreen.

Lipase Assay

A modified version of the lipase assay reported by Johnson *et al.* was used(174). Briefly, overnight cultures of *A. baumannii* strains were cultured in LB broth. The lipase activity of each culture was measured by a spectrophotometer after addition of 0.9 mM of the substrate, 4-nitrophenyl myristate in 80 mM Tris/0.15% Triton X-100 buffer. The absorbance at 415 nm was measured over time at 37°C. All assays were performed in triplicate with means and standard deviations presented.

Results

Recent studies have demonstrated the significance of the T2SS in colonization by *A. baumannii* and *A. nosocomialis* in murine models of bacteremia and pneumonia

(174-176). In our study, we also revealed that one of the secreted proteins, LipA, contributes to colonization (174), possibly by aiding in nutrient acquisition through lipid hydrolysis. It is quite likely that other T2S substrates including the lipase LipH, the phospholipase LipAN, and/or other putative enzymes and proteases identified by proteomics similarly contribute to *in vivo* survival of *A. baumannii* (175, 176).

In addition, a “serum resistance factor” may be secreted by the T2SS, as a previous study aimed at identifying factors that contribute to *A. baumannii* proliferation in human serum identified a *gspN* transposon insertion mutant with diminished serum survival (31). Here, we followed up on this finding by testing the possibility that an intact T2SS is required for *A. baumannii* ATCC 17978 to resist serum complement. Many isolates of *A. baumannii* survive in the presence of 100% serum; however, ATCC 17978 is sensitive to this concentration; therefore, we conducted our experiments using 50% serum. The WT and Δ*gspD* mutant strains were incubated in 50% pooled human sera, and the CFUs were determined after 30 min incubation at 37°C. As a control, we used the Δ*lipA* mutant that has an intact T2SS but lacks one of the T2S substrates, LipA. We also treated the WT and mutant strains with heat-inactivated (HI; 56°C, 30 min) serum, in which the complement system is inactivated. While no loss of viability was observed for the WT and Δ*lipA* strains, only 1% of the Δ*gspD* mutant cells survived 30 min in normal serum (Figure 4.2A). Next, we subjected the Δ*gspD* mutant cells to factor C1q-depleted and factor B-depleted human sera. The majority of Δ*gspD* mutant cells survived in the absence of factor B, which is required for activation of the alternative complement pathway; however, in the C1q-depleted serum, which is deficient in the classical pathway yet contains factor B, less than 0.05% of the Δ*gspD* mutant cells were

viable (Figure 4.2B). This result suggests that the T2SS directs the outer membrane translocation of a factor that provides protection from the alternative pathway. In contrast, the Δ*gspN* mutant was not affected by human serum (Figure 4.2A), a result that differs from the study published by Jacobs *et al.* (31). The lack of a serum sensitive phenotype for the Δ*gspN* mutant is consistent with our earlier finding that GspN is not required for T2S in *A. baumannii* (174) and with the discovery by Wang *et al*. that showed GspN is not needed for survival of *A. baumannii* in a mouse model of pneumonia (172). We suggest, therefore, that the diminished growth observed for the *gspN* transposon mutant in human serum is due to a polar effect of the transposon on the downstream gene, *gspD*, which we show here is required for full protection from serum complement. Taken together with earlier findings, these results support the model that extracellular proteins secreted by the T2SS play important roles in the pathogenesis of *A. baumannii* and suggest that the T2SS may be an attractive target for therapeutic intervention.

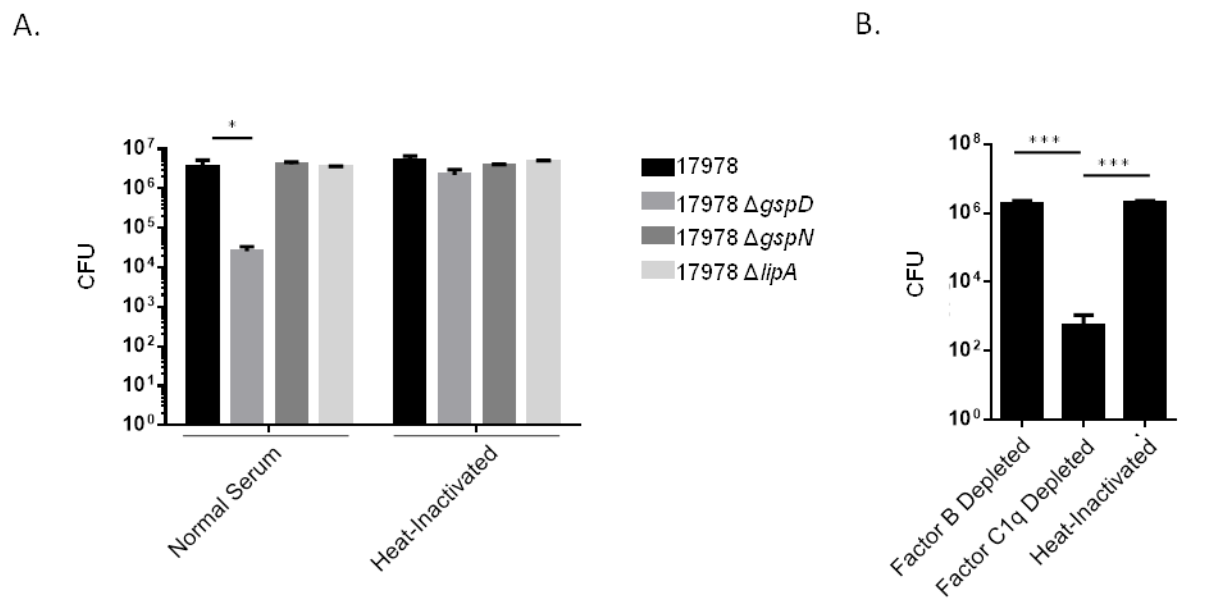


Figure 4.2. Survival in serum depends on the T2SS. A. Cells from overnight cultures were washed and incubated for 30 min at 37° C with either 50% normal human serum or heat-inactivated serum. Cells were plated for CFUs following incubation. * $p<0.05$ by Student t-test; n=3. **B.** Cells from overnight cultures were washed and incubated as above with normal human serum, Factor B deficient, C1q deficient, or heat-inactivated serum. n=3, *** $p<0.001$ by Student t-test.

Inhibiting the function of the T2SS would simultaneously prevent the secretion of many substrates, causing a greater impact on *A. baumannii* survival than targeting individual T2S substrates. The T2SS components and their interactions (Figure 4.1) provide ideal targets for therapeutics because they are unique to the T2SS and are absent from most members of the human microbiota. Compounds could block the function of the outer membrane pore, inhibit interactions between the different components of the secretion apparatus, prevent recognition of the substrates by the apparatus, or interfere with the expression of the T2S proteins (Figure 4.1). To identify inhibitors of the T2SS, we developed a novel HTS approach.

Development of High Throughput Assay

In our previous study, we used a colorimetric lipase assay to measure the activity of the T2S lipase LipA in culture supernatant of strains overexpressing plasmid encoded *lipBA* genes. We used an overexpression strain because endogenous lipase production is very low during growth of *A. baumannii* ATCC 17978 in LB, presenting a challenge for detection (174). Further, because lipase activity is undetectable in the culture supernatant of T2SS mutants, this provides a robust assay that can be used as a readout for T2SS activity (174) for the purpose of identifying T2SS inhibitors. However, testing the effect of a large number of compounds on LipA activity in cell-free culture supernatants would be cumbersome, as it would involve an extra processing step. Thus, we compared cell-free culture supernatants and unfractionated cultures for T2SS activity (Figure 4.3).

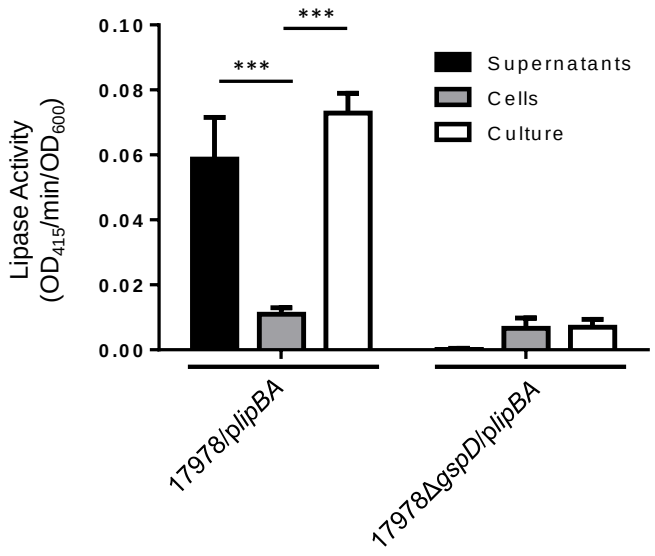


Figure 4.3. Detection of T2S lipase activity in either supernatants separated from cells or unfractionated bacterial cultures. Enzymatic activity of either the supernatant, cells, or unfractionated cultures of 17978/p*lipBA* or 17978Δ*gspD*/p*lipBA* strains grown overnight in 5 mL LB broth. The substrate 4-nitrophenyl-myristate was added to the samples and the change in OD_{415} was recorded over time. Values were normalized to the OD_{600} of the original cultures. *** $p < 0.001$ by Two Way Anova.

The culture supernatant and unfractionated culture of the 17978/p*lipBA* strain both contained significant lipase activity toward 4-nitrophenyl myristate, while there was little to no activity either in the supernatant or culture of the T2SS mutant, 17978Δ*gspD*/p*lipBA*. More importantly, the vast majority of the lipase activity in the unfractionated culture was generated by extracellular LipA, thus allowing us to omit the step in which the culture supernatant is separated from cells. Consequently, we developed an assay for HTS of small molecule inhibitors of T2SS in which the lipase substrate was added directly to the bacterial cultures following growth in the presence and absence of compounds.

In our first attempt to miniaturize the assay we grew cultures of the WT 17978 strain and Δ*gspD* mutant in the wells of clear 96-well microtiter plates to which we then

added 4-nitrophenyl myristate and determined the lipase activity by measuring the increase in absorbance at 415 nm over time. Prior to adding the lipase substrate, we measured the density of the cultures at 600 nm. This is an important step because many compounds, including known antibiotics, affect the growth of the bacteria resulting in false positives. While the move to the microtiter format required titration of IPTG to induce *lipBA* expression, a reproducible difference in lipase activity could be measured in the WT and mutant cultures. We further developed the lipase secretion assay in flat-bottom 384-well plates using a MicromultiDrop liquid dispenser. As the conditions developed for the 96-well plates did not generate reproducible data in the 384-well format, we set up a systematic analysis to evaluate a variety of conditions to obtain the most consistent data. We varied growth media, culture volume, IPTG concentration, starting OD_{600} of the culture, growth temperature, length of growth, aeration, and finally, 4-nitrophenyl myristate concentrations (Table 4.1). The experimental protocol that yielded the most consistent growth and reproducible lipase activity from well-to-well and plate-to-plate employed cultures grown in 10 µL MH broth (from a starting OD_{600} of 0.005) with 50 µM IPTG in a humidified chamber at 24°C without shaking for 16 h (Table 4.1).

Table 4.1. Optimization of assay conditions for development of a High-Throughput Screen.

Conditions Optimized	Values Tested	Final Assay Condition
Plate format	96 well vs 384 well	384 well
Start OD	0.005, 0.025, 0.001, 0.0005	0.005
Media	LB vs Mueller-Hinton (MH)	MH
Volume	10, 15, 20, or 30 µL	10 µL
Temperature	20°, 24° (RT), 30°, 37°	RT
Aeration	Shaking vs non-shaking	Non-shaking
Substrate Concentration	0.225, 0.45, 0.9, 1.8 mM	0.45 mM

Pilot Screen

Following optimization, we screened 6,400 pharmacologically active compounds as well as FDA-approved drugs from the following libraries available at the University of Michigan Center for Chemical Genomics: MS2400, Prestwick, LOPAC, BioFocus NCC, and Focused Collections. This pilot screen evaluated the strength of the assay before moving on to larger compound libraries. Each 384-well plate contained 320 sample wells, 32 positive control wells, and 32 negative control wells. As the ultimate goal of the HTS is to identify T2SS inhibitors, the T2S mutant, 17978Δ*gspD*/p*lipBA* served as our positive control, and 17978/p*lipBA* served as the negative control. Both negative and positive controls were cultured in the presence of 0.5% DMSO, whereby the sample wells containing 17978/p*lipBA* received the compounds resuspended in DMSO yielding a 0.5% final DMSO concentration. Following growth, the absorbance at 600 nm was measured for the cultures in each well. The average OD_{600} for the negative and positive controls were 0.21 ± 0.02 and 0.19 ± 0.02, respectively. The lipase substrate was then

added, and the absorbance at 415 nm was measured over a period of 20 min at ambient temperature. The pilot screen yielded a z-factor of 0.65 [$z' = 1-(3*(\sigma_p + \sigma_n)/(|\mu_p-\mu_n|))$](204) (Figure 4.4A) and coefficient of variation (CV, $CV=\sigma/\mu$) of 0.03 and 0.07 for the negative and positive controls, respectively. Initial active compounds were identified using statistical comparisons to positive and negative controls present on every plate. In the triage process, we selected compounds that resulted in all of the following: a reduction in lipase activity that was equal or greater than 3 SD of the negative control, a minimum cut-off at 30% inhibition of lipase activity and an OD_{600} value greater than 0.17 (Figure 4.4). Implementing these criteria yielded 191 compounds (3% hit rate). From these, we removed 22 compounds that are known antibiotics and retested the remaining 169 compounds.

Each compound was re-tested twice in a concentration-dependent manner using the original DMSO stock and covering two orders of magnitude. IC_{50} values were calculated. Forty-eight compounds gave IC_{50} values of <30 µM. Following removal of compounds that affected growth, 34 active compounds remained (0.5% hit rate). Fresh powder of these compounds was ordered and retested. Of these compounds, 21 were confirmed as active. The compounds with the lowest IC_{50} values are known lipase inhibitors, Orlistat and Ebelactone B, and therefore likely had a direct effect on the lipase activity itself. Orlistat, a pancreatic lipase inhibitor, was the most potent of the compounds tested with an IC_{50} of 40.6 nM (Figure 4.5A). The other compounds exhibited IC_{50}s between 4.3 and 27 µM (Table 4.2). The titration curves of Orlistat (Figure 4.5A) and Oxyclozanide (Figure 4.5B) are shown as examples. Of note, compounds with low IC_{50} values included tricyclic antidepressants that are known to act

as serotonin-norepinephrine re-uptake inhibitors. While these latter compounds may act on the secretion system, it is possible they also bind directly to the lipase via their hydrophobic rings. Our proof-of-concept pilot screen with z'=0.65 and CV of 0.03 and 0.07 for the negative and positive controls, respectively, showed that our assay was reproducible and was capable of identifying compounds that result in a statistically significant reduction in lipase activity. However, the identification of compounds that are known lipase inhibitors emphasized the importance of developing counter screens and other follow-up assays to remove false positives and to assure specificity to identify inhibitors of the T2SS, not its substrates.

A.

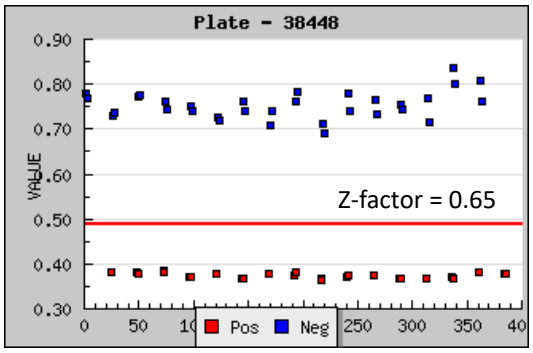
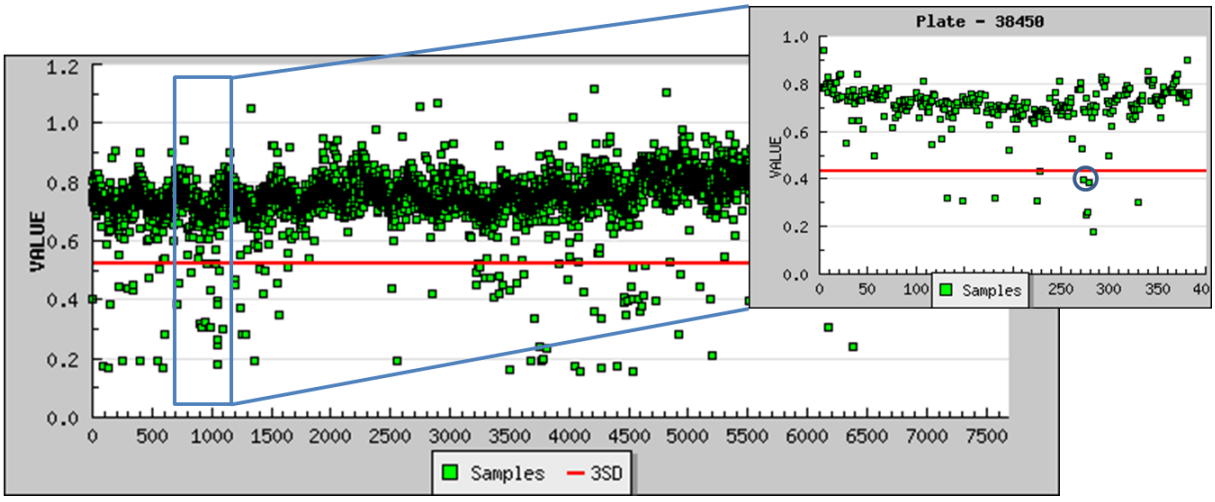


Figure 4.4. Demonstrating the feasibility of the lipase assay for high-throughput screen. A. One sample plate from the pilot screen showing the OD_{415} values for the positive (red) and negative (blue) controls**.** The z-factor for the entire pilot assay was calculated using $1-(3*(\sigma_p + \sigma_n)/(|\mu_p-\mu_n|))$ where σ is the standard deviation and μ is the mean. **B.** The results for 6400 compounds tested in the pilot screen. A single sample plate is highlighted in the inset. All samples below the red line (3 SD from negative controls) were taken into consideration. The compound Orlistat, indicated by the circle, represents a hit in the primary screen and was tested further.

B.



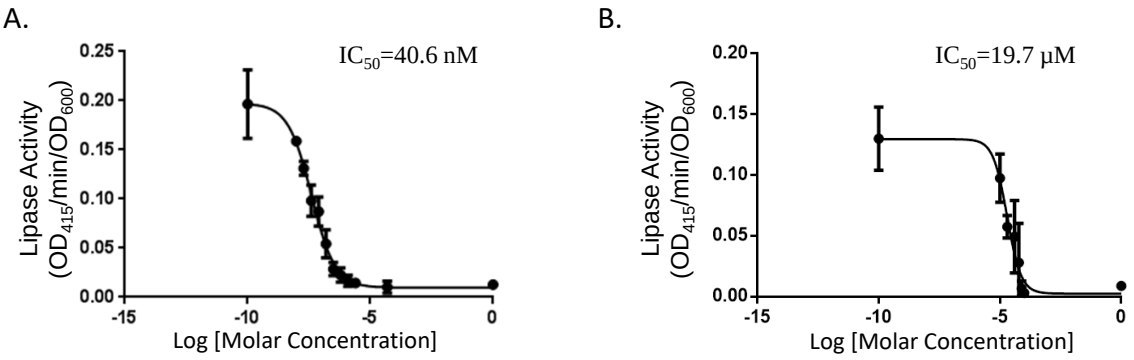


Figure 4.5. Titrations of compounds and the corresponding IC50. A. Titration of Orlistat, a pancreatic lipase inhibitor identified in the primary screen. Increasing amounts of Orlistat was included during growth of 17978/p*lipBA*. After overnight incubation in 96-well plates, the substrate 4-nitrophenyl myristate was added, and the lipase activity was measured. Values have been normalized to OD600 of culture. n=3, bars represent standard deviation from the mean. **B.** Titration of Oxyclozanide, an antihelmintic that was identified in the primary screen. Lipase activity in the presence of Oxyclozanide was measured as above. Values have been normalized to OD600 of culture. n=3, bars represent standard deviation from the mean.

Table 4.2. IC_{50} values of compounds identified in primary screen and tested for concentration dependent inhibition.

Compound	**IC_{50} (µM)**
Lipase Inhibitors	
Ebelactone B (n=2)	4.3
Anti-Depressants	
Vivactil	10
Fluoxetine	10.5
Aventyl	14.5
Duloxetine	16
Norcyclobenzaprine	16
Maprotiline	16
Desiprimine	16.5
Lofepramine	21
Sertraline	23.5
Other	
Perhexiline Maleate	4.7
Febuxostat	8
Alfuzosin	11
Stattic	12.5
Fendiline	13
Tomoxetine	14.5
Indatraline	27

Counter screening

As the most potent compounds identified in our pilot screen described above represented lipase inhibitors, we developed a screen to eliminate these types of compounds. In this counter screen, the bacterial cultures were not grown in the presence of compounds. Instead, a large batch of 17978p/*lipBA* culture was grown without compounds, and, following removal of cells, the cell-free culture supernatant containing the lipase was distributed in 384-well plates and incubated with the compounds, thus allowing us to identify compounds that inhibit the lipase itself. Following optimization, we identified the following conditions for the counter screen: 1) grow 17978p/*lipBA* with 50 µM IPTG for 16 h and remove cells by centrifugation; 2) dilute the supernatant 1:10 and add 10 µL to 384-well plates containing compounds; 3) add 10 µL 4-nitrophenyl myristate at 0.45 mM, incubate 10 min, and measure the change in absorbance at 415 nm.

To eliminate compounds that interfere with plasmid-encoded *lipBA* expression, an additional counter screen was developed. For this purpose, we used the same plasmid backbone as p*lipBA* but replaced the *lipBA* gene with the *gfp* gene, which codes for Green Fluorescent Protein (p*gfp*). After introducing this plasmid into the WT 17978 strain (17978/p*gfp*) and without lysing the cells, reproducible GFP fluorescence was significantly higher than the fluorescence detected for 17978 with the vector alone negative control (17978/p) (Figure 4.6). We optimized the conditions and applied them in the following counter screen: cultures were grown in 10 µL MH from a starting OD_{600} of 0.005 with 75 µM IPTG in a humid chamber at 24°C without shaking for 16 h and using 17978/p*gfp* and 17978/p as negative and positive controls, respectively. For this

counter screen, the compounds would be added to the cultures at the start of growth, with the fluorescence measured after growth. Any compound that is positive in both the primary screen and this counter screen is likely targeting expression of the plasmid-encoded lipase and should be removed from the pool of potential hits.

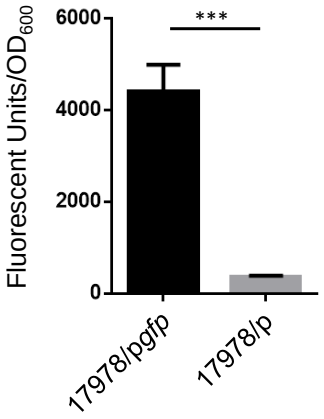


Figure 4.6. Detection of GFP fluorescence. Strains of 17978/p and 17978/p*gfp* were grown overnight in the presence of IPTG to induce the expression of GFP. After growth, fluorescence of intact cells of each strain was measured at 485 nm excitation, 520 nm emission and normalized to the OD=600 of the cultures. n=3, *** p<0.001 by Student t-test.

Twenty-one compounds that had responded in a concentration-dependent manner in the primary HTS and then confirmed when using fresh powders were subjected to the lipase inhibitor and GFP counter screens. All of these compounds were identified as either lipase inhibitors (n=18; Table 4.2 and Figure 4.5A) or inhibitors of *lipBA* expression from the plasmid (n=4; representative compound shown in Figure 4.5B), highlighting the necessity of utilizing counter screens before pursuing detailed characterization of false positives.

As we move forward to screen larger libraries to identify T2SS inhibitors, our protocol will involve the following order of analysis (Figure 4.7). First, the active

compounds from the primary HTS (step 1) will be delivered in triplicate to three different sets of 384-well plates using the original DMSO stock solutions (step 2). The first set of plates will represent a repetition of the primary screen. The second and third sets of plates will be used to counter screen for compounds that inhibit lipase activity or plasmid-borne gene expression, respectively (Figure 4.7). Compounds that are positive in the counter screens will be eliminated from further consideration, and the remaining compounds will be tested for their ability to prevent secretion over a range of concentrations (step 3). Fresh compounds will be reordered and tested (steps 4 and 5). Active compounds will then be analyzed for inhibition of secretion in additional *A. baumannii* strains, as ideal T2SS inhibitors should be active against the T2SS of all the *A. baumannii* isolates regardless of antibiotic resistance phenotype.

We have begun to test lipase activity of other strains of *A. baumannii* that were isolated from different body sites, are resistant to different antibiotics and produce different amounts of biofilm (Figure 4.8). While we have previously shown that detection of lipase activity in the culture supernatant of ATCC 17978 grown in LB in the absence of lipids requires overexpression of LipA from a plasmid, other strains display lipase activity without the need for LipA overexpression (Figure 4.8). The difference in lipase activity among the strains may be due to differences in expression of *lipA* as well as the presence of other lipases such as LipH which may or may not be dependent on the T2SS for extracellular secretion. We constructed a T2SS mutant of one of the strains, AB031 (to be described elsewhere). This Δ*gspD* mutant had a statistically significant reduction in lipase activity compared to the WT AB031 strain, indicating that a detectable portion of the lipase activity stems from a T2SS-dependent lipase(s) (Figure

4.8). This strain, as well as any others we may find as we continue to screen *A. baumannii* isolates for extracellular lipase activity, may be used to further analyze active hits to help determine which compounds to pursue. Analysis of additional strains such as AB0057 and AB5075 that show low lipase activity, however, will likely involve plasmid-expression of LipA.

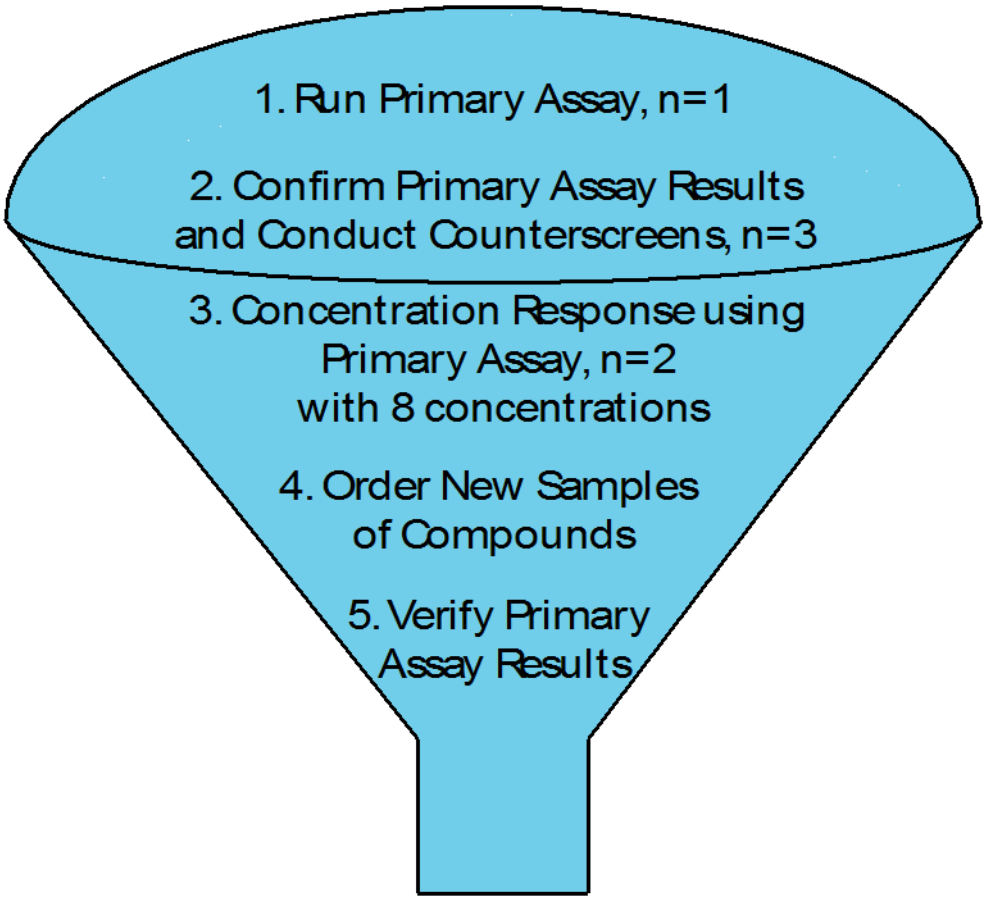


Figure 4.7. High-Throughput Screening Schematic.

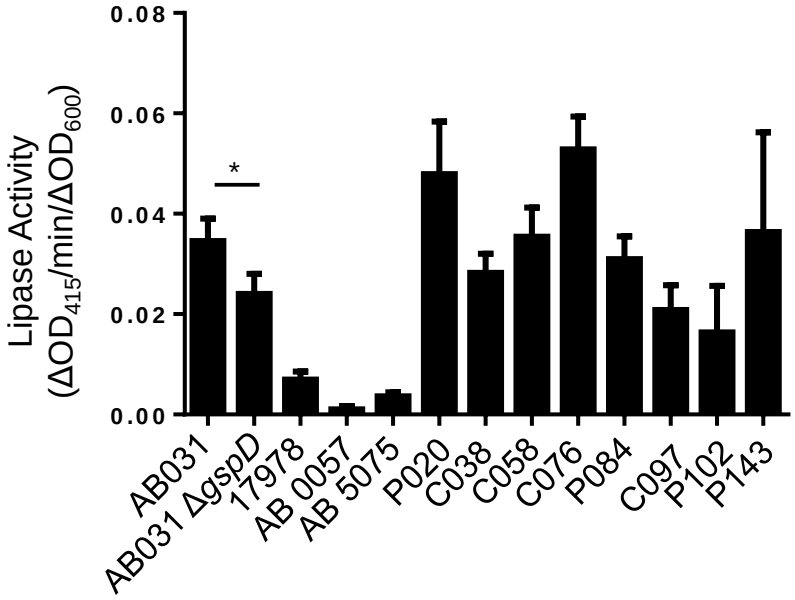


Figure 4.8. T2S lipase activity of other *A. baumannii* strains. The lipase substrate 4-nitrophenyl myristate was added to overnight cultures of the indicated *A. baumannii* strains, and the lipase activity was measured. Values were normalized to the OD_{600} readings of each culture. Bars show standard deviations from the means, n=3. * $p<0.05$ by Student t-test.

Discussion

Here, we provide additional information on the T2SS in *A. baumannii*. In addition to supporting colonization, in part through the secretion of LipA, we show that the T2SS also contributes to serum resistance, as there is a ≥100-fold reduction in recoverable CFUs of ATCC 17978 Δ*gspD* mutant following exposure to human complement. The mechanism by which the T2SS protects *A. baumannii* from human complement is not known but published reports have shown that *A. baumannii* expresses secreted and surface-associated proteins that contribute to serum resistance, and it is possible that they use the T2SS for outer membrane translocation. The serine protease PKF is produced with an N-terminal signal peptide, a prerequisite for T2S, and CipA, another serum resistance factor is a predicted lipoprotein (27, 30). Lipoproteins, such as

pullulanase and SslE, are examples of surface-associated T2S substrates, and CipA may similarly localize to the cell surface via the T2SS (79, 205). While PKF is a member of the HtrA family of chaperone-proteases that refold or degrade misfolded proteins in the periplasmic compartment (206), it is detected in *A. baumannii* culture supernatant, possibly due to its secretion via the T2SS.

The reduced *in vivo* fitness in mouse models of bacteremia (174) and pneumonia (176) and the increased sensitivity to complement killing of T2SS deficient *A. baumannii* (Figure 4.2) suggest that the T2SS plays an important part during infection through the action of specific T2S substrates, and thus shows great potential for therapeutic targeting. GspD may be an especially promising target, as it forms a gated channel in the outer membrane through which transport occurs. Potential drugs, therefore, would not need to penetrate membranes to reach their target and would not be subject to the effect of drug efflux pumps. This latter point may be of particular interest in the treatment of bacteremic *A. baumannii*, as the expression of several efflux pumps are upregulated when *A. baumannii* is cultured in human serum (207).

Here, we describe the development and optimization of a HTS to identify small molecule inhibitors of the T2SS in *A. baumannii*. Using a previously published assay, we developed, optimized, and tested a high-throughput assay on a small library of pharmacologically active compounds. Our assay demonstrated little fluctuations within controls and showed an acceptable dynamic range between our positive and negative controls yielding a z-factor of 0.65. Our proof-of-concept pilot study also indicated that the assay is simple, straightforward, reproducible, robust, and specific as it identified all four lipase inhibitors and seven tricyclic antidepressants present among the 6,400

compounds tested. In addition, the pilot study underscored the importance of including counter screens to reduce the number of false-positives that target substrates, but not the T2SS. We feel confident that our primary lipase screen and counter screens have been sufficiently optimized to be used to screen larger libraries of compounds.

Other studies have implemented similar approaches for the identification of secretion inhibitors with notable differences. The first screen developed, validated and used a gain-of-signal screen to identify inhibitors of SecA, an essential component of the Sec export pathway in *Pseudomonas aeruginosa* (208). No inhibitors were identified that directly interfere with the Sec pathway, as the transport of the periplasmic enzyme β-lactamase was not affected. However, following application of secondary assays, a set of compounds was found to reduce the extracellular activity of T2SS substrates, although they had no effect on the secreted substrates themselves, suggesting that the compounds inhibit their secretion (208). Our screen differs from the Sec screen in that we use a T2SS mutant as our positive control, thus increasing the specificity of our assay. In addition, our screen includes high-throughput counter screens that remove false positives early in the triage process. The screen developed and validated by Tran *et al.* utilized the plant pathogen *Dickeya dadantii* (209). Similar to our screen, the authors measured OD_{600} after growth to detect antibiotics and used the activity of a T2S substrate to measure T2SS function. As with the Moir *et al.* screen, the Tran *et al.* screen did not utilize counter screens in the high-throughput screening process (208, 209). What is apparent in all three studies is that identified compounds have to be subjected to many additional tests before they can be verified as true T2SS inhibitors. Among others, the presence of active compounds should cause an accumulation of T2S

substrates in the periplasmic compartment. Once we implement our screen for the identification of T2SS inhibitors in *A. baumannii*, it will be advantageous to compare any active compounds to the compounds discovered in these two other screens to search for similarities amongst the compounds.

While the primary goal of this pilot screen was to develop a HTS regimen to identify compounds to target the T2SS, our data indicate that our dual screen combined with counter screens also have the potential to identify compounds with antibiotic properties and reveal new targets for known pharmacological compounds already in use. For example, we discovered that the tricyclic antidepressants efficiently inhibit *A. baumannii* LipA. Less surprising was the finding that the pancreatic lipase inhibitors Orlistat and Ebelactone B efficiently inhibit LipA activity. However, as our previous study has shown that LipA enhances *A. baumannii* colonization (174) and that *A. baumannii* secretes several lipolytic enzymes including LipH and LipAN that may also support *in vivo* survival of *A. baumannii* (175, 176), a lipase inhibitor has potential for therapeutic use. Along with T2SS inhibitors, our HTS may also identify inhibitors of LipB, the chaperone for both LipA and another T2S substrate, LipH (175), which may also be developed for therapeutic intervention.

While a T2SS inhibitor for therapeutic purposes is our ultimate goal, we are also interested in pursuing small molecules that can be used as tools to study T2S in multidrug resistant strains as they are often genetically intractable and very difficult to study systematically. Therefore, development of chemical probes to advance virulence studies of these antibiotic resistant isolates is also critically important.

Table 4.3. List of Bacterial Strains and Plasmids used in this study.

Strain or Plasmid	Characteristics	Source or Study
Strain		
17978	WT for this study	ATCC
17978Δ*gspD*	T2S mutant	(174)
17978Δ*gspN*		(174)
17978Δ*lipA*		(174)
AB031	Clinical strain	(177)
AB031 Δ*gspD*	T2S mutant	Waack et al., in preparation.
AB 0057	Clinical strain, tet^R, chl^R, $trim^R$, $carb^R$	(168)
AB 5075	Clinical strain, tet^R, rif^R, $carb^R$, $trim^R$	(169)
P020	Clinical strain, cef^R	(210)
C038	Clinical strain, cef^R	(210)
C058	Clinical strain, mer^R, azt^R, cef^R, cip^R, lev^R, imi^R	(210)
C076	Clinical strain, azt^R, cef^R, cip^R, lev^R, $trim^R$	(210)
P084	Clinical strain, mer^R, azt^R, cef^R, cip^R, lev^R, imi^R	(210)
C097	Clinical strain	(210)
P102	Clinical strain, mer^R, azt^R, cef^R, cip^R, lev^R, imi^R	(210)
P143	Clinical strain, azt^R, cef^R	(210)
Plasmid		
pMMB67EH	Low copy vector (Ap^r)	(163)
p*lipBA*	pMMB67EH-*lipBA* (Ap^r)	(174)
p*gfp*	pMMB67EH-*gfp* (Ap^r)	(211)

Chapter V:

Discussion

Due to the escalating prevalence of antibiotic resistant infections caused by *A. baumannii*, this nosocomial pathogen is receiving increased attention. While much research has focused on the mechanisms of antibiotic resistance, there is a growing interest in the mechanisms of *A. bauamanii* pathogenesis. Like many opportunistic pathogens, a single virulence factor, such as a toxin, does not appear to cause all or even the majority of disease symptoms. Instead, *A. baumannii* seems to thrive due to a variety of mechanisms, some of which were described in Chapter I. The goal of my dissertation research was to increase our understanding of the pathogenesis of *A. baumannii* by investigating the contributions by the T2SS.

Discovery of the T2SS in *A. baumannii*

In Chapter II, I presented our study that highlighted the presence of a functional T2SS in *A. baumannii* which is notable due to the unique organization of the T2S genes in multiple operons instead of the typical one or two (Figure 2.1). We demonstrated that wild-type *A. baumannii* but not a T2SS mutant is able to grow on lipid agar (Figure 2.2). In addition, we identified a lipase, LipA, which is responsible for this growth. Both a LipA mutant and a T2SS mutant were outcompeted by the isogenic wild-type strain when

colonizing either the murine liver or the spleen following intravenous inoculation (Figure 2.6). This suggests that both the T2SS and LipA promote *A. baumannii* colonization in the bacteremia model.

One interesting avenue of research that stems from this study would be to understand the unique genetic organization of the T2SS genes in *A. baumannii* (Figure 2.1). As mentioned in Chapter II, the genes for the T2SS are generally encoded in one or two operons while the T2SS genes in *A. baumannii* are spread over several operons throughout the genome. This raises the question of what drove the scattered genetic organization of *A. baumannii*. Were the genes originally in one or two operons like other T2SS systems but have separated into multiple small operons over time? Alternatively, did the genes integrate into the genome in different places at different times when the bacterium acquired the T2SS genes from other bacteria? I believe the first hypothesis or some version of it is likely the correct explanation. There do not appear to be any insertion sequences near the T2SS genes that would indicate an insertion of those genes. In addition, obtaining the genes in a piecemeal manner does not seem advantageous for the bacterium, since at a minimum, all the core genes (*gspC-M* and *pilD*) are needed to gain any known advantage for retaining any of the genes. Currently, all sequenced *A. baumannii* strains possess all the T2SS core genes, which are highly homologous, an observation that indicates the full set of genes were acquired before the strains diverged from each other. Future work could take a bioinformatics approach to address the questions: What does the organization of the T2SS genes look like in the

Acinetobacter genus? Do any *Acinetobacter* species lack the genes or contain only a subset of the genes?

In addition to the questions raised by the genetic organization of the T2SS genes in *A. baumannii*, our first study also leaves unanswered questions regarding the lipase LipA. We hypothesized that LipA might be utilized for nutrient acquisition, interference with host cell signaling, or for an unknown function. Interestingly, *A. baumannii* secretes several lipases, including a phospholipase via the T2SS (174-176). The lipases may work together to acquire nutrients, possibly by targeting host cell membranes. It may be surprising that loss of a single secreted substrate reduced the ability of the *lipA* mutant strain to compete with the wild-type strain (Figure 2.6). One would think that the mutant strain would benefit from the activity of the lipase secreted by the wild-type strain as long as it is in proximity at high cell density. This would be the case with either nutrient acquisition or interference with host cell signaling. If the lipase is breaking down long-chain fatty acids, the bacterium closest to the breakdown product would benefit, but it would not necessarily be the same bacterium that secreted the lipase. The same would be true if the lipase interferes with host cell signaling. However, at low cell density the wild-type and mutant cells are likely present in clonal populations in which the mutant cells will not be in close contact with wild-type cells. It is also possible that the wild-type and mutant cells are not occupying the same location within the organs. Both strains must survive in the blood stream before colonizing either the spleen or the liver. If the strains do not arrive in the same area of the organs, it would explain why the *lipA* mutant is not able to benefit from the LipA secreted from the wild-type strain.

The T2SS, CpaA, and fXII

In Chapter III, we investigated the function of another T2S substrate, CpaA, in a different strain of *A. baumannii*, AB031. Previous work by other groups has shown that the metalloprotease CpaA increases the time to form blood clots as measured by the aPTT assay(177). In addition, CpaA is T2-secreted and requires a putative chaperone, CpaB for folding and/or secretion(175). We have expanded on these two studies and shown that a Δ*cpaA* mutant is less competitive than a wild-type strain in a murine model for bacteremia (Figure 3.3). In addition, we demonstrated that CpaA cleaves the clotting factor fXII in a heavily glycosylated proline-rich domain between Pro308 and Thr309. By deglycosylating fXII before incubation with CpaA, we showed that the glycosylation of fXII is necessary for cleavage by CpaA but not for activity of fXII.

The requirement of fXII glycosylation for cleavage by CpaA is particularly interesting, especially as there are individuals without a glycosylated T309 due to a mutation. As mentioned in Chapter III, the T309K and T309R mutations are found in patients with hereditary angioedema Type III. Patients with other forms of hereditary angioedema have mutations in C1-inhibitor, which represses the cleavage and autoactivation of fXII (212). Thus, these patients have an unchecked activation of the kallikrein/kinin system. Patients with hereditary angioedema Type III have the same symptoms and increased activation of the kallikrein/kinin pathway but normal C1 inhibitor function and an overactive fXII caused by the mutations. These mutations are autosomal dominant and, although rare, can be tracked through families. Are the mutations protective in some manner and the increased activation of fXII and potentially

life-threatening outcome reasonable side effects? Perhaps the answer lies in its host's ability to survive infections from bacteria such as *A. baumannii*. The normal host response relies on the innate immune system and can include trapping the bacterium in a clot to prevent dissemination and present an easy target for immune cells. To circumvent this natural defense mechanism, we postulate that *A. baumannii* secretes CpaA to prevent or prolong the formation of clots. Perhaps host fXII mutations are a way to counter act this virulence strategy. Conversely, it is possible that there is no advantage to having these mutations for survival but instead there has not been sufficient negative selection to remove the fXII mutations from the gene pool.

There are several ways to determine which hypothesis is the correct one. From an epidemiological standpoint, we could compare the rates of infection of *A. baumannii* in individuals with hereditary angioedema type III versus healthy individuals. This approach may be problematic due the small number of individuals with this condition. Ideally, we would also compare the different types of hereditary angioedema and determine if the rate of infection changes depending on which mutation a patient has. Another possible method is to perform mouse studies, although this approach has its own problems. As shown in Figure 3.4, CpaA increased the clotting time for both human and murine plasma, but was less potent in the murine plasma. This pattern may indicate substrate specificity, since murine fXII and human fXII are only 70% identical. In addition, the site of CpaA cleavage in human and murine fXII is not identical (Figure 5.1). Thus, a humanized mouse model may facilitate these studies.

```
FA12_MOUSE      MTALLFLGSLLMSLDLTLSAPPWKDSKKFKDAPDGPTVVLTVDGRLCHFPFQYHRQLHHK
                M ALL LG LL+SL+ TLS PPW+  K+ K   +  TVVLTV G  CHFPFQYHRQL+HK
FA12_HUMAN      MRALLLLGFLLVSLESTLSIPPWEAPKEHKYKAEEHTVVLTVTGEPCHFPFQYHRQLYHK

FA12_MOUSE      CIHKRRPGSRPWCATTPNFDEDQQWGYCLEPKKVKDHCSKHNPCHKGGTCINTPNGPHCL
                C HK RPG +PWCATTPNFD+DQ+WGYCLEPKKVKDHCSKH+PC KGGTC+N P+GPHCL
FA12_HUMAN      CTHKGRPGPQPWCATTPNFDQDQRWGYCLEPKKVKDHCSKHSPCQKGGTCVNMPSGPHCL

FA12_MOUSE      CPEHLTGKHCQKEKCFEPQLLKFFHENELWFRTGPGGVARCECKGSEAHCKPVASQACSI
                CP+HLTG HCQKEKCFEPQLL+FFH+NE+W+RT    VARC+CKG +AHC+ +ASQAC
FA12_HUMAN      CPQHLTGNHCQKEKCFEPQLLRFFHKNEIWYRTEQAAVARCQCKGPDAHCQRLASQACRT

FA12_MOUSE      NPCLNGGSCLLVEDHPLCRCPTGYTGYFCDLDLWATCYEGRGLSYRGQAGTTQSGAPCQR
                NPCL+GG CL VE H LC CP GYTG FCD+D  A+CY+GRGLSYRG A TT SGAPCQ
FA12_HUMAN      NPCLHGGRCLEVEGHRLCHCPVGYTGAFCDVDTKASCYDGRGLSYRGLARTTLSGAPCQP

FA12_MOUSE      WTVEATYRNMTEKQALSWGLGHHAFCRNPDNDTRPWCFVWSGDRLSWDYCGLEQCQTPTF
                W  EATYRN+T +QA +WGLG HAFCRNPDND RPWCFV + DRLSW+YC L QCQTPT
FA12_HUMAN      WASEATYRNVTAEQARNWGLGGHAFCRNPDNDIRPWCFVLNRDRLSWEYCDLAQCQTPTQ

FA12_MOUSE      APLVVPES----------QEESPSQAPSLSHAPNDST--------DHQTSLSKTNTMGCG
                A    P S          Q   P   P+    P   T        +   SL++   + CG
FA12_HUMAN      AAPPTPVSPRLHVPLMPAQPAPPKPQPTTRTPPQSQTPGALPAKREQPPSLTRNGPLSCG

FA12_MOUSE      QRFRKGLSSFMRVVGGLVALPGSHPYIAALYWGNNFCAGSLIAPCWVLTAAHCLQNRPAP
                QR RK LSS  RVVGGLVAL G+HPYIAALYWG++FCAGSLIAPCWVLTAAHCLQ+RPAP
FA12_HUMAN      QRLRKSLSSMTRVVGGLVALRGAHPYIAALYWGHSFCAGSLIAPCWVLTAAHCLQDRPAP

FA12_MOUSE      EELTVVLGQDRHNQSCEWCQTLAVRSYRLHEGFSSITYQHDLALLRLQESKTNSCAILSP
                E+LTVVLGQ+R N SCE CQTLAVRSYRLHE FS ++YQHDLALLRLQE    SCA+LSP
FA12_HUMAN      EDLTVVLGQERRNHSCEPCQTLAVRSYRLHEAFSPVSYQHDLALLRLQEDADGSCALLSP

FA12_MOUSE      HVQPVCLPSGAAPPSETVLCEVAGWGHQFEGAEEYSTFLQEAQVPFIALDRCSNSNVHGD
                +VQPVCLPSGAA PSET LC+VAGWGHQFEGAEEY++FLQEAQVPF++L+RCS  +VHG
FA12_HUMAN      YVQPVCLPSGAARPSETTLCQVAGWGHQFEGAEEYASFLQEAQVPFLSLERCSAPDVHGS

FA12_MOUSE      AILPGMLCAGFLEGGTDACQGDSGGPLVCEEGTAEHQLTLRGVISWGSGCGDRNKPGVYT
                +ILPGMLCAGFLEGGTDACQGDSGGPLVCE+  AE +LTL+G+ISWGSGCGDRNKPGVYT
FA12_HUMAN      SILPGMLCAGFLEGGTDACQGDSGGPLVCEDQAAERRLTLQGIISWGSGCGDRNKPGVYT

FA12_MOUSE      DVANYLAWIQKHIAS
                DVA YLAWI++H  S
FA12_HUMAN      DVAYYLAWIREHTVS
```

Figure 5.1. Amino acid alignment of murine fXII and human fXII. The highlighted residue is the O-linked glycosylated T309 that is important for CpaA cleavage. The underlined sequence is the sequence identified by N-terminal sequencing in chapter three. Note the lack of homology between the human and mouse fXII in that region.

During our study, we expressed recombinant histidine-tagged wild-type, T309K, and T309R fXII in a cell line derived from human kidney. Although we visualized expression of all three proteins via Western blotting and purified each by cobalt affinity chromatography (not shown), we were unable to determine cleavage of any of them by CpaA, including the wild-type protein. Additionally, we noticed that none of the

recombinant proteins were detected with a monoclonal fXII antibody, although they were efficiently recognized by polyclonal fXII antibodies, suggesting that these variants may differ from plasma-derived fXII in posttranslational modification such as glycosylation (data not shown). We are attempting other methods to produce recombinant fXII that has the correct fXII glycosylation needed for recognition by CpaA. Alternatively, we could procure plasma from patients with hereditary angioedema type III for analysis.

CpaA cleaves fXII (Figure 3.7A) as well as fV (Figure 3.7B and reference (177)), even though only the cleavage of fXII results in inactivation. Do these two proteins share a similar motif or glycosylation pattern that CpaA recognizes? One way to begin to answer this question is to cleave fV with CpaA and then to submit the cleaved products for N-terminal sequencing. This strategy would determine the cleavage site in fV. If this cleavage site also has O-linked glycosylated residues, it could explain why fV is also cleaved by CpaA, although the physiological ramifications, if any, would be unknown.

Another lingering question is what is the physiological consequence of CpaA mediated cleavage of fXII? Under normal circumstances, fXII is cleaved when activated (Figure 5.2, blue triangle), but the domains are still connected via a cysteine bond (labeled as A in Figure 5.2). This cysteine bond is cleaved and the two domains are separated. CpaA's cleavage site is not located within the catalytically active domain (Figure 5.3, enlarged inset) and would, theoretically, have no effect on the fXII activity. However, cleavage of fXII separates the heavy chain from the catalytic domain

prematurely and may thus prevent surface binding, an important function of the heavy chain, and thus autoactivation of fXII.

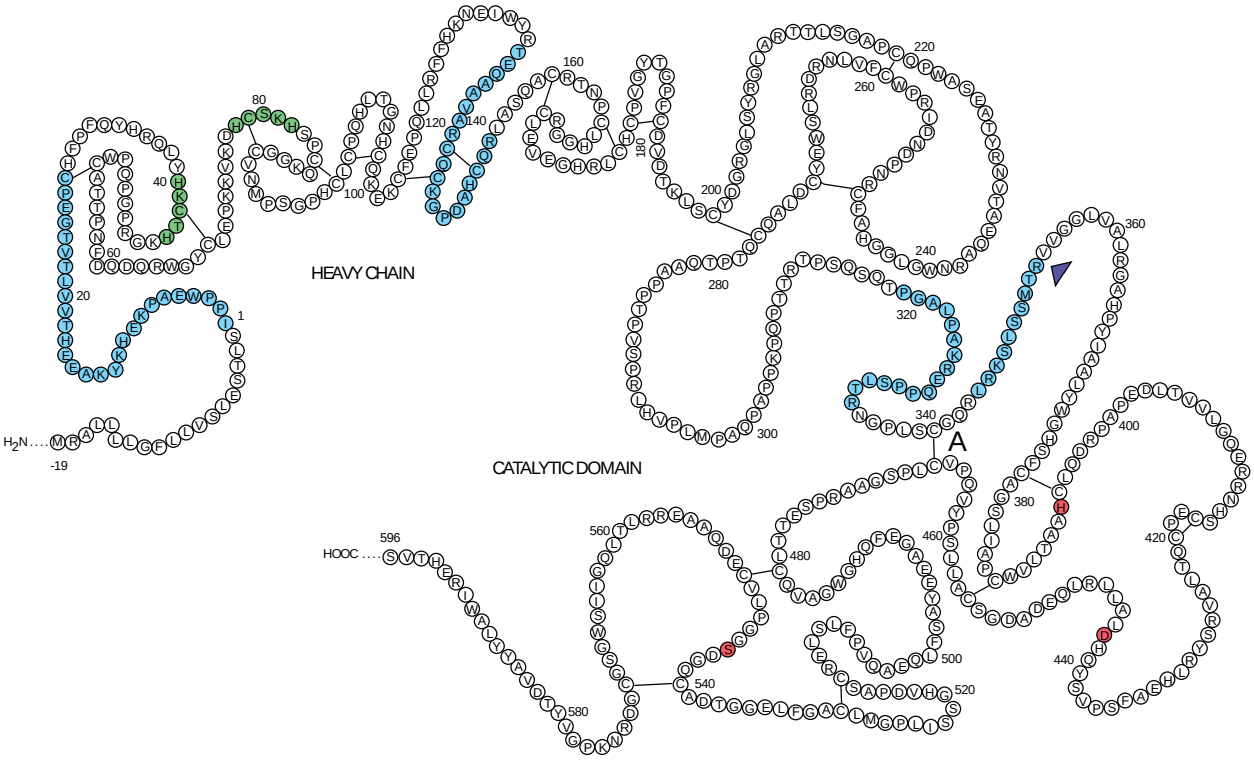


Figure 5.2. Cringle diagram of fXII. Amino acids colored blue encode a surface binding site. Green amino acids comprise a zinc-binding domain. The catalytic residues are represented by red amino acids. Cysteine bonds are depicted by a straight line. The letter A indicates the cysteine bond that connects the heavy chain and catalytic domain after activation. The blue triangle represents the cleavage site for fXII activation. Figure modified from Colman and Schmaier (213).

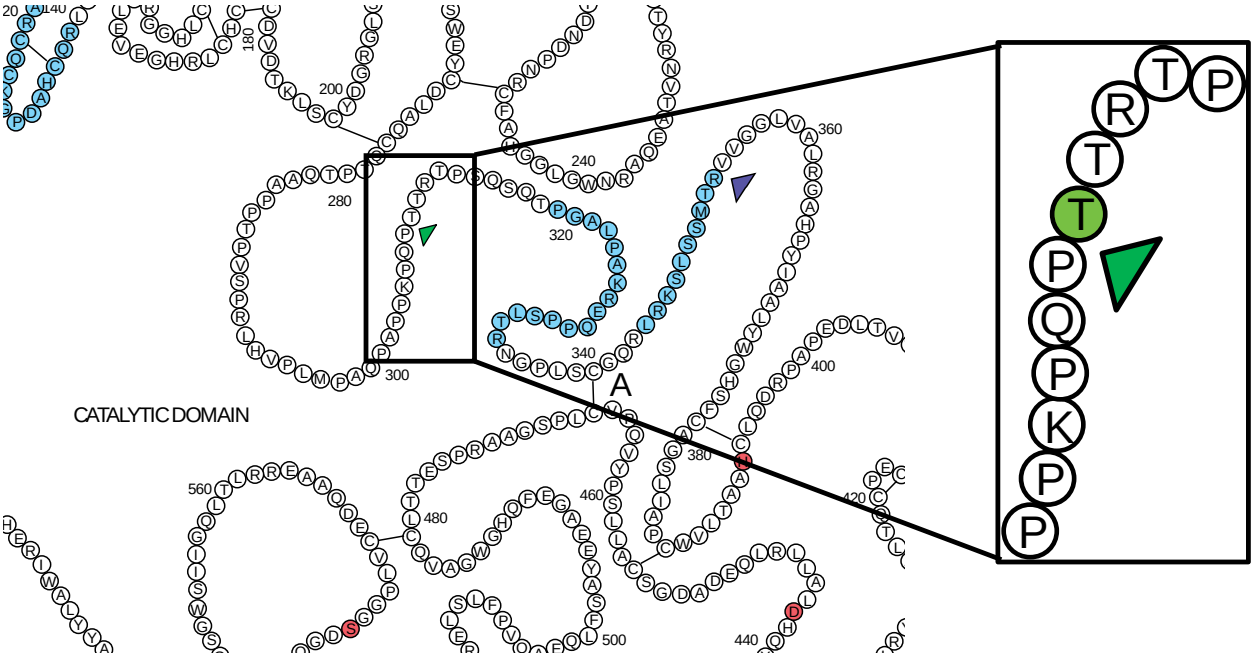


Figure 5.3. Cleavage Site of CpaA. The cleavage site of CpaA (shown by green arrow) is shown in the enlarged view of the fXII sequence. CpaA cleaves before T309, indicated by the green amino acid. The site of cleavage for activated fXII is shown by the blue arrow, and A indicates the cysteine bond which connects the heavy and catalytic domains. Figure modified from Colman and Schmaier (213).

Future work should examine whether the T309K and T309R mutations in fXII prevent CpaA mediated cleavage, investigate the specificity of CpaA, and determine the physiological implications of CpaA cleavage of fXII.

Targeting the T2SS

My last project attacked the *A. baumannii* pathogenesis conundrum from a different angle. While both chapters II and III contribute to our understanding of the role of the T2SS in pathogenesis by examining the function of particular secreted substrates, chapter IV aimed to find inhibitors of the T2SS. We know from both our work and that of others that the T2SS is required for colonization of *A. baumannii* and the closely related *A. nosocomialis* in both a bacteremia and pneumonia model in mice (174, 176) as well as in a greater wax moth model, *Galleria mellonella* (175). In addition, we showed that

the *A. baumannii* strain ATCC 17978 requires a T2SS but not the secreted lipase LipA to withstand complement-mediated killing (Figure 4.2). This pattern suggests that the T2SS secretes many substrates which could promote colonization and survival. The potential to prevent secretion of many substrates as well as its multitude of potential drug targets makes the T2SS a promising therapeutic target. To this end, we developed, optimized, and validated a high-throughput lipase assay to identify T2SS inhibitors. In addition, we developed and optimized two counter screens to minimize potential false positives. We showed that a robust, specific, and sensitive assay with counter screens is now ready to be utilized to test larger libraries of compounds.

Following compound library screening, our list of potential inhibitors will be greatly reduced when we move forward with additional specificity assays. For example, we can use other *A. baumannii* strains with different antibiotic resistance patterns to test for lipase activity and only proceed with compounds that have an effect on several strains. To allay concerns that the inhibitor only inactivates lipase and/or its chaperone, we will use the clotting assay described in Chapter III to test the compounds for interference with CpaA secretion. In addition, we will also perform tests designed to verify that our compounds do indeed inhibit the T2SS. For example, when the T2SS is no longer active, the substrates accumulate in the periplasm(126). The same phenomena should occur with LipA and CpaA as well as other T2S substrates after treatment of the bacterium with the compounds.

Once we are confident that the compounds indeed target the T2SS, we can move our compounds forward. By utilizing the resources available at the University of

Michigan, we can perform structure-activity relationship analysis to develop and produce compound(s) with higher activity. In addition, studies will investigate the mechanism of inhibition and identify the precise target of the compound(s). Any promising compounds will also be tested for pharmacological properties and efficacy *in vivo*. Furthermore, even compounds that may not be useful as a drug could be utilized as research tools. For example, compounds could be used to characterize the structure and function of the T2SS. Many strains of *A. baumannii* are resistant to a wide variety of antibiotics, and it would be difficult to genetically manipulate these strains to construct T2SS mutants using canonical cloning techniques. However, by using a compound to inhibit the T2SS *in vitro*, we could gain insight into the role of the T2SS in these strains.

Concluding Remarks

My dissertation has presented novel work on the T2SS and its role in the pathogenesis of *A. baumannii*. We have demonstrated the activity of the T2SS in *A. baumannii* as well as probed the activity of its two substrates, LipA and CpaA. We have shown that the T2SS as well as LipA and CpaA support *A. baumannii* colonization in a murine bacteremia model. We characterized CpaA and showed that it cleaves fXII and demonstrated that O-linked glycosylated residue(s) are necessary for this cleavage. Furthermore, we developed, optimized, and validated a high-throughput screening assay to identify small molecule inhibitors of the T2SS. This assay has been proven to be robust, sensitive, and reliable. Taken together, my dissertation has highlighted the importance of the T2SS in the pathogenesis of a highly concerning hospital acquired pathogen and made progress toward the isolation of potential therapeutic agents to prevent and treat *A. baumannii* infections.

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